From SECTION OF OPHTHALMOLOGY AND VISION
DEPARTMENT OF CLINICAL NEUROSCIENCE
S:T ERIK'S EYE HOSPITAL
Karolinska Institutet, Stockholm, Sweden

INTERACTIONS BETWEEN NEURAL RETINA, RETINAL EPITHELIUM AND CHOROID

by

Lena Ivert

Karolinska Institutet

Stockholm 2006

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7140-797-9

To my dear mother

You never gave up hope and kept on encouraging me so that this book came true despite everything.

In dear memory of my father.

Life is really simple,
but we insist making it so complicated.
Confucius

ABSTRACT

The retinal pigment epithelium (RPE) is a non-replicating monolayer that plays a key role nursing the photoreceptors of the neural retina, regulating fluid movement within the subretinal space, maintaining a basal laminar layer of Bruch's membrane, and influencing the choriocapillaris. This project investigates how surgical manipulation of the retinal epithelial layer influences the neural retina, the choroid and the epithelial layer itself.

Bleb detachment is a surgical method to gain access to the subretinal space by separating the neural retina from the RPE, which can be therapeutically useful in procedures such as gene therapy and retinal cell transplantation. This method of separating the RPE from the photoreceptors is not without complications because it can produce a number of abnormalities in the RPE itself as well as in the neural retina and choroid. Jet stream pressure, the standard way to produce a bleb detachment, causes focal damage to the RPE layer and choroid and generalized damage to the apical membranes of the RPE. It also produces folds in the rabbit neural retina, which induce a gradual transformation of the RPE layer. This transformation leads to migration, proliferation and a defect in lysosomal digestion that has been associated with apoptosis. Such changes produced by bleb detachment may explain the gradual loss of RPE allografts or homografts.

Photoreceptor transplants survive for a long period of time and can develop outer segments without showing evidence of apoptosis. But such photoreceptor transplants form abnormal rosettes, which may also be due to abnormalities produced in the RPE layer by bleb detachments. These problems associated with the production of bleb detachments should be considered in any attempts to introduce solutions and/or cells into the subretinal space.

Attempts to reconstruct or repair the RPE layer by transplantation can involve the removal of RPE cells from Bruch's membrane before replacing them with transplanted cells; it can also involve biopsy of RPE cells for culture or transplantation to another site. The removal of RPE cells from Bruch's membrane causes significant changes in the choroid. Removal of large areas of RPE leads to inflammation and fibrosis in the choroid that compresses the large choroidal vessels leading to reduction of blood flow in the terminal choriocapillary beds. The absence of an RPE layer without its replacement may also cause atrophy of the choriocapillaris by the loss of a hypothetical trophic factor. Even slight pressure on the RPE layer without any removal of cells leads to rapid but reversible reduction in flow of large choroidal vessels, which appears to be due to vasospastic constriction and/or thrombosis. Because the recovery from such changes can take hours or days, this abnormality could lead to degeneration of the neural retina and may be related to other choroidal abnormalities.

Removal of local segments of the neural retina causes profound changes in the adjacent RPE layer, which are even more pronounced than what is observed in bleb detachments or what has been described after prolonged retinal detachments. It reveals that the neural retina must inhibit the transformation of the RPE layer by releasing a factor that suppresses this response.

The results are important for understanding how to enter and manipulate the structures bordering the subretinal space, the photoreceptors, the retinal epithelium and the choroid. It offers insights into how the methodology for surgical repair of these structures such as gene therapy and/or transplantation can be made more effective.

Keywords: RPE, neural retina, basal lamina, Bruch's membrane, choroid, subretinal space, bleb detachment, ICG, FAG, SLO, choroidal blood flow.

LIST OF PUBLICATIONS

The thesis is based on the following original publications. They will be referred to by their Roman numerals in the text. The papers are reprinted with the permission from Springer Verlag.

I. Ivert L, Gouras P, Naeser P, Narfström K (1998) Photoreceptor allografts in a feline model of retinal degeneration. Graefe's Archive Clin Exp Ophthalmol 236: 844-852.

II. Ivert L, Kjeldbye H, Gouras P (2002) Long-term effects of short-term retinal bleb detachments in rabbits. Graefe´s Archive Clin Exp Ophthalmol 240: 232-237.

III. Ivert L, Kong J, Gouras P (2003) Changes in the choroidal circulation of rabbit following RPE removal. Graefe´s Archive Clin Exp Ophthalmol 241: 656-666

IV. Ivert L, Kong J, Gouras P (2006) Alteration in choroidal blood flow produced by local pressure. Graefe´s Archive Clin Exp Ophthalmol March 17:1-6 (Epub ahead of print)

V. Ivert L, Gouras P (2006) Behavior of retinal epithelium to bleb detachment versus retinectomy. Graefe´s Archive Clin Exp Ophthalmol April 26:1-6 (Epub ahead of print)

VI. Ivert L, Kjeldbye H, Gouras P (2005) Age related changes in the basement membrane of the retinal pigment epithelium of RPE65 -/- and wild type mice. Graefe´s Archive Clin Exp Ophthalmol 243: 250-256

CONTENTS

LIST OF ABBREVIATIONS

APMPPE	Acute posterior multifocal placoid pigment epitheliopathy
BM	Basement membrane
BM40	Basement membrane protein 40
BSS	Balanced salt solution
CHS	Chediak-Higashi Syndrome
CRABP	Cellular retinoic acid binding protein (Amacrine & Műller cells)
CRALBP	Cellular retinaldehyde binding protein or Cis-retinaldehyde binding protein (in RPE & Műller cells)
CRBP	Cellular retinol binding protein (in Műller cells)
CSC	Central serous chorioretinopathy
EM	Electron microscopy
FAG	Fluorescein angiography
HSPGs	Heparan sulphate proteoglycans (Type XVIII collagen)
ICG	Indocyanine green angiography
IPE	Iris pigment epithelium
IPM	Interphotoreceptor matrix
IRBP	Interphotoreceptor retinoid binding protein (in IPM)
LM	Light microscopy
MEM	Minimal essential medium
Mertk	C-mer proto-oncogene tyrosine kinase A gene important for the ROS phagocytosis
MEWDS	Multiple evanescent white dot syndrome
PE	Pigment epithelium
PVR	Proliferative vitreal retinopathy
RBP	Retinol binding protein (in the blood)
RCS	Royal college of surgeons
Rd	Retinal degeneration
ROS	Rod outer segment
RP	Retinitis Pigmentosa
RPE	Retinal pigment epithelium
SLO	Scanning laser ophthalmoscopy
SPARC	Secreted protein acidic and rich in cysteine. This is a non collagenous matrix protein.
SRBP	Serum retinol binding protein
VMD2	Vitelliform macular degeneration 2 gene

1 INTRODUCTION

1.1 RETINAL EPITHELIUM, BRUCH'S MEMBRANE, CHOROID AND NEURAL RETINA

The retinal epithelium plays a critical role in maintaining the integrity and function of both the neural retina and choroid. On the one hand, it acts as a nursing cell to the photoreceptors providing them with key molecules involved in photo-transduction of light into neural signals, in phagocytizing and digesting the continuous shedding of photoreceptor outer segments and in controlling the milieu of the subretinal space. On the other hand, it is also thought to influence the choroidal circulation by sending trophic signals that maintain the integrity of the choriocapillaris, which in several pathological states can lead to choroidal neovascularization. The epithelium also influences Bruch's membrane of the choroid by continuing to synthesize basal lamina, the innermost component of this acellular structure. In addition, the neural retina acts to prevent any migration and proliferation of the retinal pigment epithelium (RPE). These three very different cell systems, the photoreceptors, the retinal pigment epithelium and the choroid are mutually dependent on each other (figure 1).

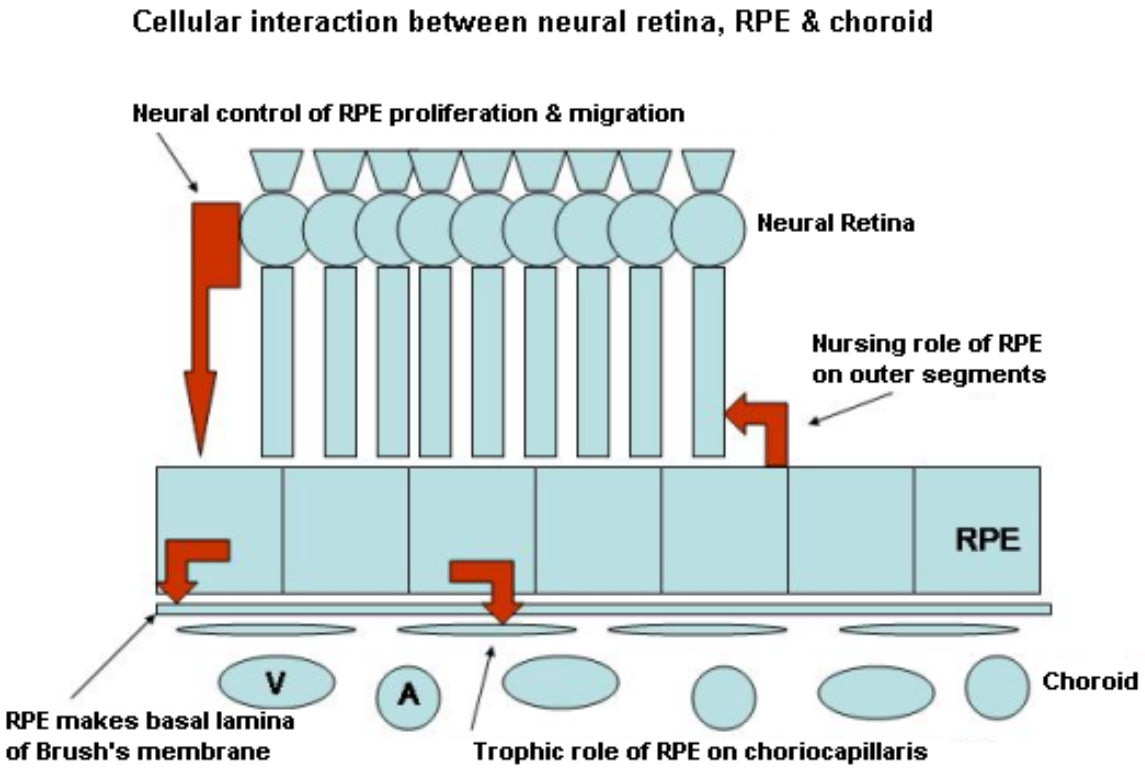

Figure 1 *shows the cellular interactions between Neural Retina, RPE and Choroid.*
V – vein
A – artery

1.1.1 The Retinal Epithelial Cell Layer

The retinal epithelium (figure 2) is a monolayer of polarized cells (Marmor & Wolfensberger, 1998). At their apical side there are multiple micro-villous processes that engulf the outer segments of the photoreceptor cells. Within these processes are ellipsoidal melanin granules that are considered to minimize the effects of scattered light. Between these micro-villous processes and the outer segments is a matrix of proteins and glycoproteins including a unique retinoid binding protein, inter-photoreceptor retinoid binding protein (IRBP) that is thought to facilitate the movements of retinol and retinal between the photoreceptors and the retinal epithelium. These villous processes are involved in phagocytizing the effete outer tips of the growing outer segments of both rods and cones. This phagocytic process is mediated by a receptor, the Mertk protein, which is expressed on these villous processes (Feng et al, 2002) and in its absence leads to a failure of phagocytosis and degeneration of the photoreceptors. These villous processes of the retinal epithelium are also responsible for maintaining the attachment of the neural retina by interdigitating and adhering to the outer segments. Firm attachment of the neural retina to the epithelial is due to several factors: passive hydrostatic forces, active transport of subretinal fluid (Hughes et al, 1998), and the binding properties of the interphotoreceptor matrix as well as the interdigitation of the apical microvilli of the epithelium with the outer segments. The adhesion of the neural retina to the epithelial layer is crucial for vision because a detached neural retina can no longer register focused images and the detached photoreceptors eventually degenerate eliminating all vision.

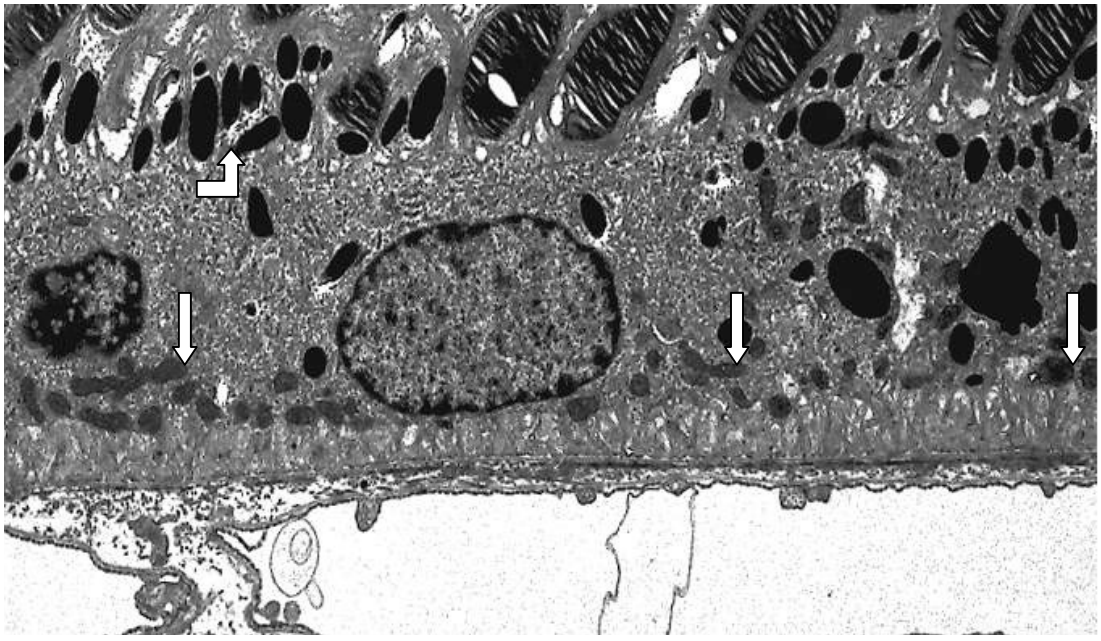

Figure 2. *Electron micrograph (EM) of a normal RPE cell with microvillous processes towards the photoreceptor outer segments. Black elliptic shaped melanin granules (bent arrow) are mainly seen in the apical processes. Mitochondria (grayish structures, straight arrows) are located close to the multiple infoldings of the basal lamina facing the choriocapillaries below.*

The basal side of the epithelium consists of multiple infoldings of the plasma membrane, which presumably facilitate the diffusion and transport of molecules from the choroid to the epithelium and vice versa (Gallemore et al, 1997). A protein, Bestrophin is a chloride channel on the basal membrane of the epithelial cell. Defects in the gene for this protein, known as the vitelliform macular degeneration 2 gene (VMD2), lead to Best's vitelliform macular degeneration (Petrukhin et al, 1998). Bestrophin is thought to regulate cell volume by regulating chloride ion movement (Fischmeister & Hartzell, 2005). The retinal epithelium appears to undergo large-scale changes in volume due to osmotic forces probably due to its active role in phagocytosis of outer segments; this chloride channel contributes to the volume regulation of the epithelial cell. The basal surface is also thought to have a possible receptor for serum retinol binding protein (SRBP) for the release and entrance of retinol into the epithelial cell.

The retinal epithelial cells are attached near their apical side by tight junctions, which restrict the passage of water and ions into the subretinal space. Additional attachments, called gap junctions, also maintain the adherence of one cell to another in the monolayer but do not restrict diffusion. Several pathological processes, including retinal detachment or photoreceptor degeneration as in Retinitis Pigmentosa (RP), can lead to dissolution of these junctions and migration of individual epithelial cells from the monolayer.

The retinal epithelial cell is a complex and dynamic structure with high metabolic demands involving the digestion of an enormous phagocytic load, an active and unique processing of retinoids and the regulation of water and molecular transport from and to the choroid into and out of the subretinal space. Figure 3 shows schematically the vitamin A cycle occurring in the retinal epithelium. The retinoid metabolism of this epithelium is extremely unique and important for vision. Each cell contains an enzyme, RPE 65, which is responsible for isomerizing all-trans retinol to the unstable 11-cis isomer of retinol. Other enzymes in the epithelial cell then oxidize the 11-cis retinol isomer to 11-cis retinaldehyde and the binding protein, cis retinaldehyde or cellular retinaldehyde binding protein (CRALBP) transports the 11-cis isomer to the apical membrane where it can be ultimately delivered to the outer segments.

One retinal epithelial cell nurses many outer segments. Defects in this unique retinoid metabolism lead to a variety of stationary and progressive photoreceptor degenerations. The basal cytoplasm of the epithelial contains numerous mitochondria, which must be responsible for much of the energy metabolism of these cells. The fact that these mitochondria are mainly located at the basal surface of the cell suggests that the transport of materials and fluid across this basal surface must demand considerable energy. This large collection of mitochondria may be responsible for oxidative damage that occurs with aging, especially at the basal surface of these cells, which could be responsible for age related macular degeneration.

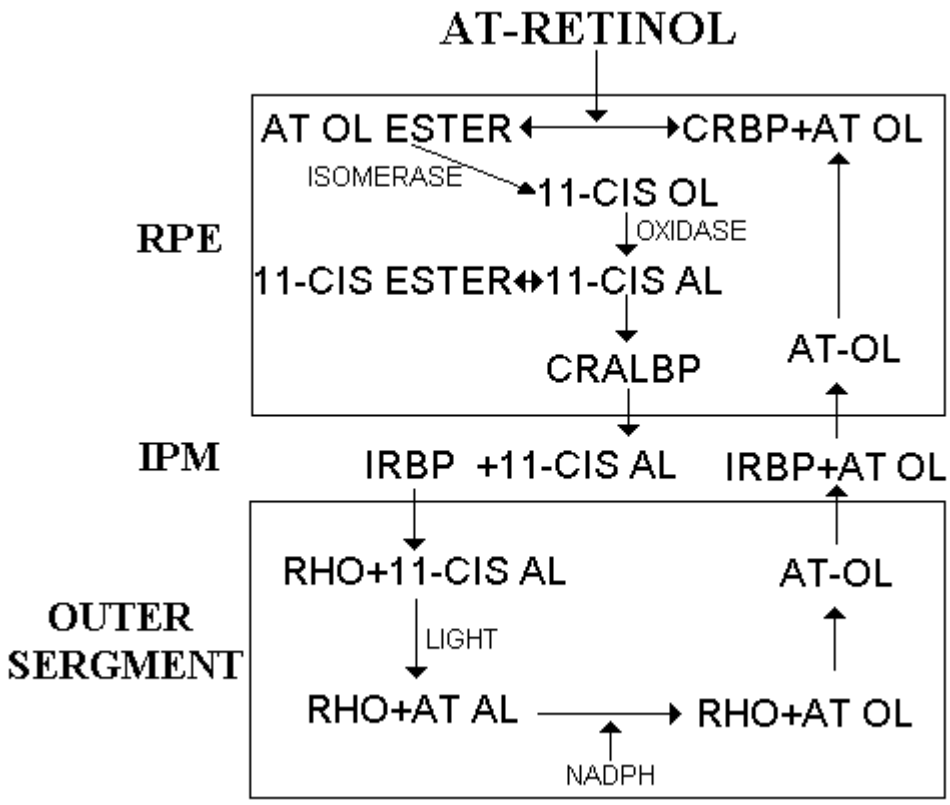

Figure 3 *shows the vitamin A cycle of vision, the Wald cycle, which involves the RPE cell and the rod. The cycle begins with all trans-retinol entering from the serum and being isomerized, oxidized and transferred to the rhodopsin in the rod outer segment. Light re-isomerizes retinal to the trans-form. It is then reduced and re-enters the RPE to complete the cycle.*
AT - all trans;
OL - retinol;
AL - retinaldehyde (retinal);
RHO - rhodopsin;
IPM - interphotoreceptor matrix;
CRBP - cellular retinol binding protein;
CRALBP - cellular retinaldehyde-binding protein;
IRBP - interphotoreceptor retinoid binding protein;

1.1.2 Bruch's Membrane

Bruch's membrane is an acellular structure about 1 μm in thickness. It is formed by five distinct layers, (1) the basal lamina of the retinal epithelium, (2) a collagen layer, (3) an elastic fiber zone, (4) an additional collagen layer and (5) the basal lamina of the endothelium of the choriocapillaris. This fine structure was discovered by Carl Bruch in 1844 (Zrenner, 1984) se figure 4 and 5.

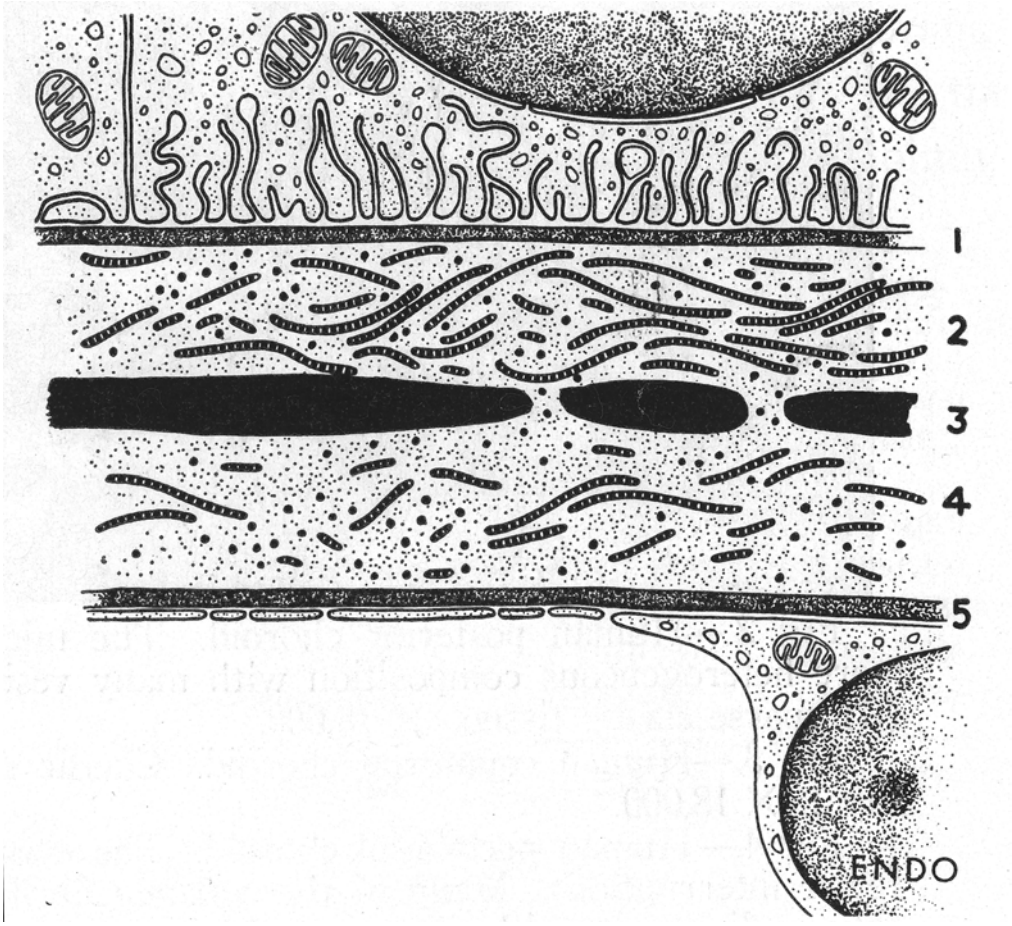

Figure 4 *shows a schematic representation of Bruch's membrane by Y Nakaizumi (1964). 1. Basement membrane of pigment epithelium; 2. inner collagenous zone; 3. interrupted elastic tissue zone; 4. outer collagenous zone; 5. basement membrane(BM) of choriocapillary endothelium. Note the basal infoldings of the plasma membrane of the PE cells and the fenestrations in choriocapillary endothelium (ENDO).*

The retinal epithelial is attached to this structure, presumably by the nature of its basal lamina. The basal lamina, sometimes also called basement membrane, are thin sheets of specialized extracellular matrix present at the epithelial/mesenchymal interface of most tissues, which surround muscle, peripheral nerve fibers, fat, endothelial and epithelial cells (Hollenberg & Burt, 1969). Originally believed to serve as a selective barrier and scaffold to which cells adhere, it has become evident that the individual components of basal lamina are regulators of biological activities such as cell growth, differentiation and migration and that they influence tissue development and repair. Although basal laminas are widespread tissue components, their fine structure and composition varies from tissue to tissue, as well as within the same tissue

at different developmental periods and during repair. All basal lamina contain laminins, entactin-1/nidogen-1, Type IV collagen and heparan sulfate proteoglycans. Some of these proteins have also been localized to extracellular matrix that lack basal lamina architecture. Perlecan is present in cartilage and some components are localized to various embryonic and reticular tissues, such as lymph nodes.

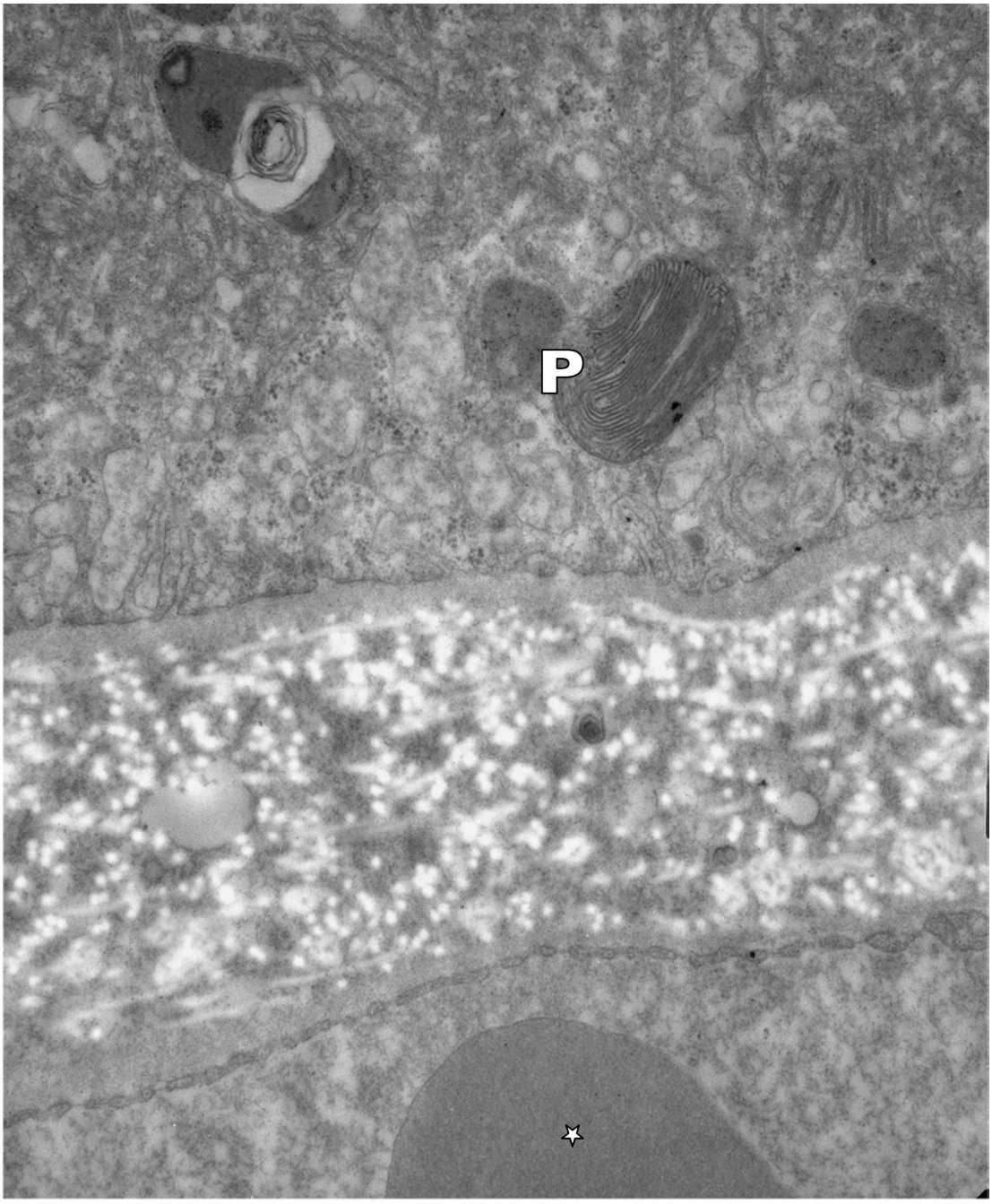

Figure 5 *shows an EM photograph of Bruch's membrane, the basal lamina of the PE, the negative staining of the collagen layer, and fenestration at the choriocapillary wall. Part of a large red blood cell (marked with a star) is seen inside the choriocapillary. In the RPE cell a phagosome (P) is seen*.

A significant number of interactions contribute to the supramolecular assembly of basal laminas. The current basal lamina model proposes two networks, one consisting of collagen Type IV and the second made up of multiple laminins,

interconnected via entactin-1. In vitro studies indicate that perlecan interacts with the other three major components through either its core protein, in the case of entactin-1 and Type IV collagen or its heparan sulfate glycosaminoglycan chains, as is the case for laminin-1. Other minor components such as BM40/SPARC/osteonectin and fibulin-1 and -2 interact with one or more of the major constituents, and these interactions may be tissue-specific, developmentally regulated and age dependent. The macromolecular nature of the basal lamina has become more complex within the past few years as more components are characterized. Recent developments in basal lamina composition and biology include the description of entactin-2/nidogen-2, characterization of agrin and collagen XVIII as heparan sulfate proteoglycans (HSPGs) and expression of the laminin family (Erickson & Couchman, 2000).

The composition of the basal lamina of the retinal epithelium is virtually unknown. There is, however, evidence that this basal lamina changes with senescence and may be critically involved in age related macular degeneration. One of the hallmarks and risk factors for age related macular degeneration is drusen, which represent small or sometimes large detachments of the retinal epithelial layer from Bruch's membrane (Figure 6).

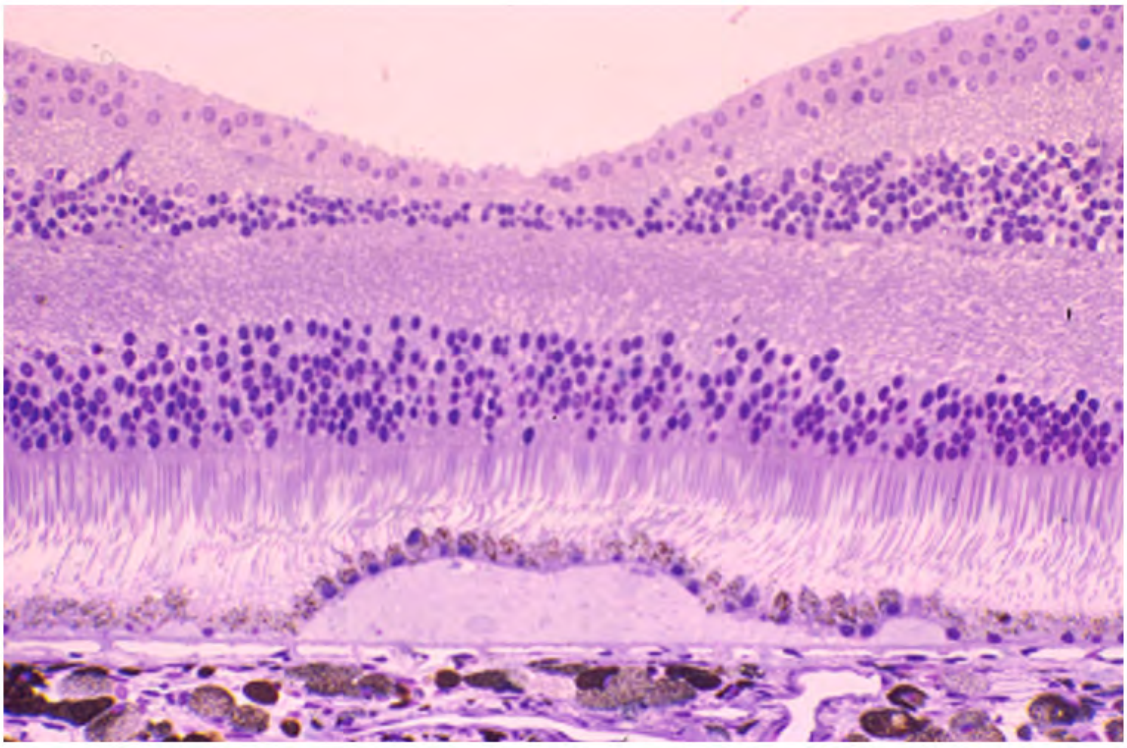

Figure 6 *shows a light microscopy (LM) photograph of a macular drusen in an aged rhesus monkey. The drusen is approximately 300 µm in extent and the elevation from Bruch's membrane is about 30 µm. There is a bulge of the outer nuclear layer over the drusen implying that the drusen may exert pressure on the neural retina. Smaller drusen are seen at both edges of the large drusen. The pigment epithelium over the drusen appears intact. The photoreceptor layer over the drusen has not degenerated as revealed by the width of the outer nuclear layer although the outer segments over the drusen are disorganized. No obvious inflammatory cells in the LM level are visible within or around the drusen. The foveal depression is visible above the large drusen.*

These detachments are produced by the deposition of material from the retinal epithelium, which occurs where the basal lamina contacts the collagen layer in Bruch's membrane. Understanding why this separation occurs at this point in Bruch's membrane can contribute to elucidating the pathogenesis of age related macular degeneration. Surgical manipulation of the retinal epithelium such as occurs in the production of bleb detachments leads to the migration and transformation of the retinal epithelium, which must presumably alter the adherence of the monolayer to the basal lamina and perhaps change the composition of this structure.

1.1.3 The Choroid

The choroid is a thin, highly vascular structure that forms the posterior aspect of the uveal tract. The capillaries or "choriocapillaris" form a unique inner monolayer that is responsible for delivering nutrients to the highly metabolic outermost neural retina. The choriocapillaris consists of end stage independent capillary beds. The basal lamina of the endothelial cells that form these capillary beds forms the outermost layer of Bruch's membrane. This endothelial basal lamina is absent between capillaries. The capillary walls formed by elongated endothelial cells are fenestrated to facilitate rapid diffusion of metabolites.

Normally no cells are found in Bruch's membrane, the structure between the choriocapillaris and RPE, but with degenerate changes in the epithelium and/or Bruch's membrane macrophages invade this otherwise acellular region. This has been found in age related macular degeneration and represents an inflammatory aspect of this disease (Hageman et al, 2001). Figure 7 shows an example from an aged primate (Gouras et al, 2006)

External to the choriocapillaris is a vascular lamina with arteries and veins that distribute blood to the capillary beds. The percentage of oxygen in the choroidal veins is about 3% of what it is in the choroidal arteries, indicating a high blood flow rate. These vessels are not auto-regulated, as retinal and brain vessels are, but are heavily innervated. External to these vessels is the suprachoroid, which contains the large vessels entering the eye. Between the choriocapillaris and the suprachoroid are a variety of cells, including melanocytes, fibroblasts and neurons and neuronal processes (Alm, 2003).

The neural innervation of the choroidal vessels is quite extensive. In the rabbit, which is the experimental animal used in our studies, nerve fibers are present in the suprachoroid and vascular lamina and are either absent or extremely rare in the choriocapillary layer. The nerve fibers can be classified as perivascular and intervascular. Perivascular fibers surround all arterial and venous blood vessels and form a network. Intravascular fibers form two groups. One group consists of fibers situated between the blood vessels and parallel to the blood vessel wall surface, so called paravascular fibers. The other group consists of fibers, which travel the entire length of the choroid until they reach the nerve plexus of the ciliary body, so called long tract fibers. Ganglion cells are also present but are small and scarce and are mostly situated in the peripheral choroid (Ramirez et al, 1999).

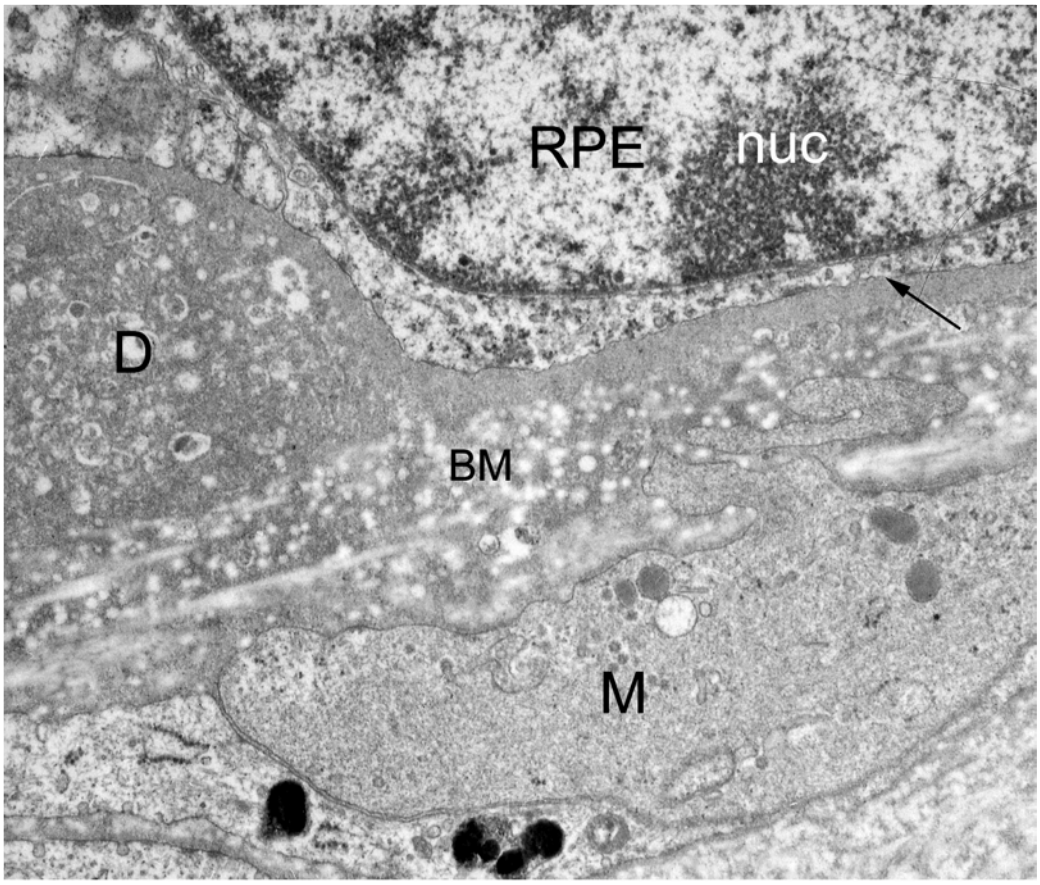

***Figure** 7 show a magnified EM photograph of an area where a macrophage has invaded Bruch's membrane (BM) close to a drusen formation (D). The basal cytoplasm below the nucleus (nuc) of an adjacent RPE cell is extremely thin and degenerating (arrow). The large segment of a macrophage (M) sends a process up to the degenerating basal cytoplasm.*

1.1.4 The Neural Retina

The neural retina is part of the central nervous system containing three distinct neuronal layers. The outermost layer contains the photoreceptor cells, rods and cones, which are polarized structures with a highly specialized outer segment that contains visual pigment molecules, opsins, which absorb light and trigger an enzymatic biochemical cascade that amplifies their response to light and alters the membrane potential of the cell. This electrical change alters the release of synaptic transmitter molecules to influence the second neural layer containing bipolar and horizontal cells. This second neural layer transmits these signals to the ganglion cell layer, which conducts this information to the brain via the optic nerve. The photoreceptors, especially cones, consume considerable metabolic energy reflected by the numerous mitochondria in the inner segments, which connect the outer segment of the photoreceptor with the cell body. The photoreceptors receive most of their oxygen from the choriocapillaris and are critically dependent on having a close association with the retinal epithelium. Any separation of the photoreceptors from the epithelial layer, as occurs in retinal detachments, leads to degeneration of the photoreceptors, which, if of sufficient duration, becomes irreversible (Fisher & Anderson, 1998).

2 CELL TRANSPLANTATIONS

2.1 RETINAL PIGMENT EPITHELIAL TRANSPLANTATION

It has been possible to transplant retinal epithelial cells into the subretinal space and in some cases to their original position on Bruch's membrane suggesting a possible therapeutic approach for abnormalities that cause degeneration or dysfunction of the retinal epithelial layer. This methodology has been successfully used to alter the course of retinal degenerations in animal models. Several laboratories have demonstrated that transplanting normal retinal epithelial cells to the subretinal space of the Royal College of Surgeons (RCS) strain of rats can prevent the photoreceptors from degenerating (Lopez et al, 1989; Li & Turner, 1988; Yamamoto et al 1993). The cause of the degeneration in this strain of rats is due to a gene defect that prevents the retinal epithelium from phagocytizing the effete outer segments. Transplants of normal retinal epithelium can restore phagocytosis and stop the retinal degeneration.

In a murine model of retinal degeneration, the RPE 65 mutant mouse, where a gene defect in the isomerization of retinol in the retinal epithelium reduces the amount of the 11-cis retinal reaching the photoreceptors, transplanting normal retinal epithelium can slow down the photoreceptor degeneration (Gouras et al, 1994). There is some indication that the transplantation of retinal epithelial cells may be handicapped by immuno-rejection (Zhang & Bok 1998).

Attempts to use this technique to influence the course of age related macular degeneration in man has not been effective, however, and this has also been attributed to host/graft rejection (Algvere et al, 1999; Kaplan et al, 1998). There are, however, other factors (Berglin et al 1997) in addition to rejection that may be influencing the outcome of subretinal epithelial cell transplants because either retinal epithelial homografts or immunosuppression cannot completely prevent such retinal epithelial transplants from degenerating (Crafoord et al, 1999; 2000 a & b; 2001). Some of the factors examined in our studies may contribute to a better understanding of this problem.

Another hypothesis has been raised to explain the degeneration of retinal epithelial transplants, especially when used in age-related macular degeneration. There is evidence that the tissue upon which the epithelial transplants are placed also determines their fate. If transplants are placed on old and therefore more degenerate Bruch's membrane, they will be eliminated by apoptosis much more than those placed on healthy Bruch's membrane (Del Priore & Tezel, 1998; Tezel et al, 1999). This implies that if a RPE layer is removed prior to transplantation, how it is removed may be important for a subsequent transplant. If the basal lamina of the host RPE layer is removed in the process, the transplant may not be provided with an ideal surface to survive.

2.2 IRIS PIGMENT EPITHELIAL TRANSPLANTATION

Iris pigment epithelial (IPE) cell transplantation has been used as a substitute for retinal pigment epithelial cell transplants because it allows for easy access of autologous cells from iris biopsies and this could eliminate the possibility of host/graft rejection. Attempts to transplant autologous iris pigment epithelium have also led to similar degeneration of the transplants in rabbits within six months (Crafoord et al, 2001). These investigators have observed a macrophagic invasion of the transplant site at six months after surgery and have suggested that melanin may be a responsible factor that initiates degeneration of these transplants. Therefore the understanding of survival and success of retinal or iris epithelial transplantation remains elusive and still poorly understood.

2.3 PHOTORECEPTOR TRANSPLANTATION

The transplantation of photoreceptors is especially intriguing because this would allow restoration of vision in a totally blind eye due to the absence of photoreceptors. Attempts to transplant photoreceptors have been tried in several different ways. The most popular method has been to transplant the entire neural retina containing the photoreceptors into the subretinal space (Ghosh et al, 1999; Gjörloff et al, 2001; Kaplan et al, 1997; Seiler et al 1999). Another method has been to dissociate the retina into small micro-aggregates containing photoreceptors (Gouras et al, 1994; Ivert et al 1998). A third way has been to attempt to remove the second and third retinal layers by sectioning or by excimer laser ablation (Salchow et al, 2001) in order to isolate a pure photoreceptor layer but this has proven to be quite difficult. There is general agreement, however that photoreceptors survive and function when transplanted to the subretinal space. There is no evidence of any host/graft rejection. The major problem with this photoreceptor or neural retinal transplantation has been the inability of such neural transplants to form functional connections with host retinal neurons.

3 GENE THERAPY

Another therapeutic approach that involves surgical manipulation of the subretinal space and its surrounding tissues is the introduction of viral solutions that can allow a gene to be placed and expressed either episomally in the cytoplasm of a cell or chromosomally in the cell nucleus where it can restore normal gene function and cure a monogenetic gene defect. This has proven effective in several animal models of retinal degeneration such as the rd mouse (Bennett et al, 1996), the RPE 65 mutant poodle dog (Acland el al, 2005) and the Stargardt mouse (Kong et al, 2005). At present there is a Stage 1 clinical trial being set up to use gene therapy to treat Leber's Congenital Amaurosis, the result of a defect in the RPE 65 gene. All of these gene therapy experiments rely on introducing viral vectors through a bleb detachment.

3.1 EX VIVO GENE THERAPY

This procedure involves transducing cultured cells with a foreign therapeutic gene and then transplanting these cells into the eye or the subretinal space (Lai et al 2000). One of the strategies for the use of this technique is to introduce trophic factors that could slow down the time course of a retinal degeneration. To avoid host/graft rejection autologous cells from the recipient could be used or the genetically engineered cells can be protected from cellular immune attack by being placed in a semi-permeable capsule.

4 BLEB DETACHMENT

All of these forms of therapy for abnormalities of the photoreceptors and/or the retinal epithelium require the use of a bleb detachment to introduce a viral solution or foreign cells. The production of bleb detachments is routinely produced by injecting a balanced salt solution contained in a glass micropipette with a flat tip diameter of approximately 50 microns or by the use of a flat, rounded 30 gauge needle. The pipette or needle is pressed gently on the surface of the neural retina and then a small amount of fluid is pressure injected through the neural retina into the subretinal space where it abruptly detaches the photoreceptors including the entire neural retina from the retinal epithelial layer. This produces a jet stream insult to the epithelial layer, the underlying Bruch's membrane and local areas of choroid. In addition, this procedure shears the outer segments from their association with the microvilli of the apical membrane of the epithelium and stretches the neural retina, especially in rabbits, which lack a retinal vasculature. The shearing frequently breaks off many of the microvilli of the retinal epithelium, which appear able to repair themselves with time. The detachment of the neural retina spontaneously reattaches over several hours. This is therefore a destructive procedure (Immel, Negi & Marmor, 1986) many of the consequences of which are examined in our studies.

5 ANGIOGRAPHY

5.1 FLUORESCEIN ANGIOGRAPHY

One of the most important tools to examine the consequences of producing bleb detachments or manipulating structures in the subretinal space is the use of angiography. Fluorescein angiography (FAG) is an older and extremely useful technique for examining retinal vasculature and the status of the retinal epithelium. Fluorescein sodium is a synthetic and highly fluorescent compound that can be injected intravenously and monitored in the retina by its fluorescence. About 60-80% of the dye is bound to serum proteins but 20 % is not and these unbound molecules freely pass through the fenestrated choriocapillaris to be blocked by an intact retinal epithelium. The entire retinal vasculature can be visualized and any defect in the retinal epithelial layer assessed by pooling of the dye under epithelial detachments or leakage into the subretinal space. What is a disadvantage of fluorescein angiography is that the excitation spectrum involves the use of blue light, which is strongly absorbed by the retinal epithelium and especially by the xanthophyll pigments in the macula. Therefore there is minimal fluorescence in the macula and little from the choroidal circulation because of the failure of blue light transmission through the pigmented epithelial layer. Another disadvantage of fluorescein angiography is that a significant amount of un-protein bound molecules enter the extracellular space of Bruch's membrane, which also blocks visibility of the choroidal vessels.

5.2 INDIOCYANINE GREEN ANGIOGRAPHY

These shortcomings of FAG are not encountered using indocyanine green (ICG) angiography. ICG is a water-soluble tricarbocyanine dye ($C_{43}H_{47}N_2NaO_6S_2$) with a molecular weight of 775 Daltons (Paumgartner, 1975). It is highly protein-bound, mainly to plasma globulins such as α_1-lipoproteins (Baker, 1966). ICG absorbs at 790-805 nm and fluoresces in the deeper infrared. Therefore it is transmitted more readily through the retinal epithelium and the macular xanthophylls pigments providing a view of the choroid and in particular the choroidal vasculature. In addition because almost all of the molecules are bound to serum proteins, there is little extravasation into Bruch's membrane from the choriocapillaris eliminating further blocking of the choroidal circulation. By means of scanning laser ophthalmoscopy (SLO), high-resolution digital imaging and double detection optics both FAG and ICG angiography can be performed simultaneously and subsequently analyzed off line supplemented by image enhancement techniques. ICG angiography in particular proved to be a valuable tool in assessing abnormalities in the choroidal circulation produced by bleb detachment and/or surgical manipulation of structures in the subretinal space. Figure 8 show the different appearance of FAG (8 C) and ICG angiography (8 D) images. In 8 C only the retinal vessels by the optic disc can be seen, while in 8 D the nice pattern of the choroidal vessels are clearly visible and it is easy to distinguish the arteries from the veins (Guyer et al, 1996)

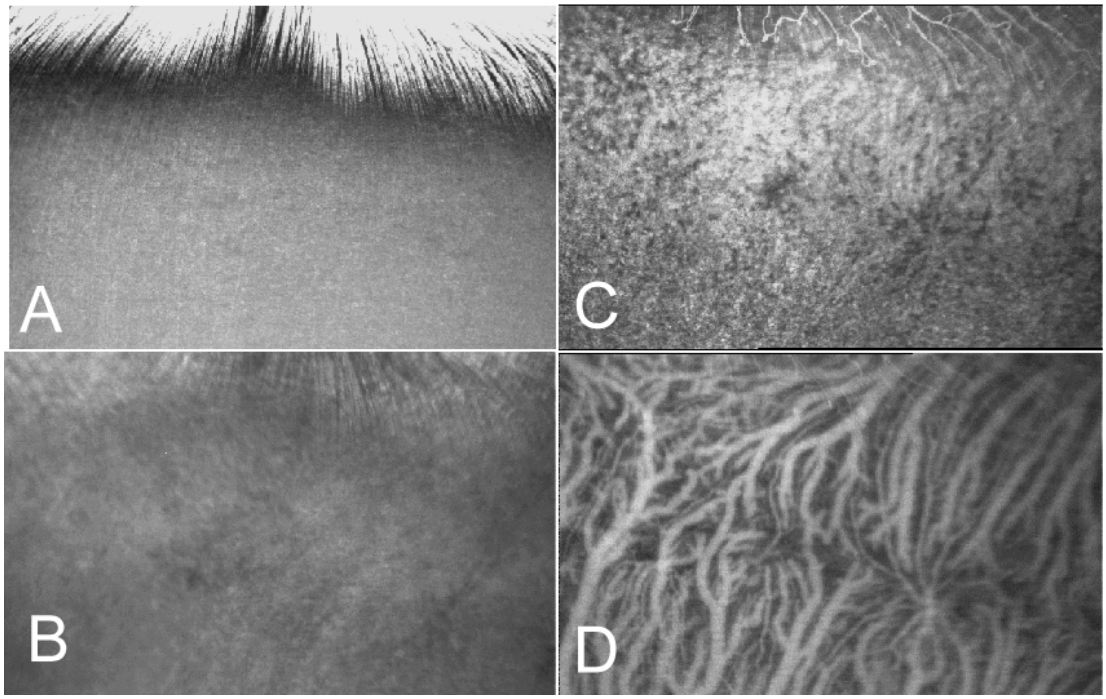

Figure 8 *show SLO images,* ***A*** *is blue at 488 nm,* ***B*** *is IR at 780 nm.* ***C*** *shows FAG (514 nm) in the midphase, diffuse fluorescence arising from the diffusion of the dye through the fenestration of the choriocapillaris into the extracellular space; no choroidal vessels can be seen.* ***D*** *is an ICG angiogram (810nm) in the midphase, which shows no diffuse fluorescence from the extracellular space but reveals the large choroidal vessels filled with the ICG dye. These SLO images are obtained from a rabbit retina, which has no retinal vessels except at the optic nerve head seen in C.*
Helium Neon laser 633 nm
Argon Laser 488 & 514 nm
Diod laser 780 nm
FAG, 488 nm is used to excite fluorescence and a green barrier filter is used for detection.
ICG, 780 nm diode laser to excite fluorescence and a longer infra-red barrier filter is used for detection of the fluorescence

6 AIMS OF THIS PROJECT

The aim of this project has been to study how surgical manipulations affect the structures bordering the subretinal space, the photoreceptors, the retinal pigment epithelium and the choroid, examining both immediate and long term changes. The reason for pursuing this research is that several therapeutic strategies such as gene therapy, cell transplantation, or the insertion of prosthetic devices, as electronic chips, require such surgical manipulations. In addition this research can reveal how these three layers of cells, the photoreceptors, the retinal pigment epithelium and the choroid, including in particular Bruch's membrane, the choriocapillaris and the larger choroidal vessels, are mutually interdependent and how changes in one can influence the others. Such research can not only contribute to our basic knowledge about the biology of these important ocular structures but also to our understanding of diseases and degenerative abnormalities that often affect these structures, such as retinal detachment, proliferative vitreo-retinopathy, age-related macular degeneration, photoreceptor degenerations and choroidal diseases.

6.1 AIMS IN THE PAPERS

1. To explore the feasibility of transplanting photoreceptor allografts into the subretinal space to replace genetically defective photoreceptors in a feline model of retinal degeneration (***Paper I***).

2. To investigate the effects of saline induced bleb detachments on the neural retina, retinal pigment epithelium and choroid (***Paper II***).

3. To investigate the effects of retinal pigment epithelium removal on the choroid and the choroidal circulation (***Paper III***).

4. To investigate how local pressure on the retinal pigment epithelial layer affects the choroidal circulation and to determine its cause (***Paper IV***).

5. To investigate the differences in the transformation of the retinal epithelial layer by bleb detachments versus local retinectomy because the former produces only a temporary while the latter results in a permanent separation of the photoreceptors from the epithelial layer (***Paper V***).

6. To determine how age and genetic status affect the basal lamina of the retinal epithelium in murine retina, comparing the RPE-/- mutant with wild type mouse (***Paper VI***).

7 MATERIALS AND METHODS

7.1 ANIMALS

Genetically defective Abyssinian cats (homozygous hereditary rod-cone degeneration), 1-3 years of age were studied in paper I to determine the feasibility of correcting photoreceptor degeneration by transplanting photoreceptor allografts into the subretinal space.

Pigmented Dutch Belted rabbits were studied in papers II, III, IV and V in the experiments designed to examine the problems associated with gaining access to the subretinal space by bleb detachments and the surgical manipulations.

Normal and genetically defective mice, RPE65-/- were studied in paper VI to examine how age and genetic status affects the retinal epithelial layer and adjacent Bruch's membrane. The RPE 65-/-mice were established in a C57Bl/6 strain and obtained from breeding pairs provided by Michael Redmond (National Institutes of Health, Bethesda, MD). Wild type C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and served as normal controls.

The RPE65 -/- knock out mouse has a genetic defect in the RPE65 gene. This defect prevents the RPE cells from synthesizing all trans-isomer to 11-cis retinol and this leads to photoreceptor degeneration. The same gene defect affects children and leads to a form of retinal blindness, called Leber's Amaurosis.

During a number of years, studies of transplanting healthy RPE cells subretinally to these mice have been carried out in the laboratory of Professor Gouras and how these transplanted RPE cells can influence and retard the photoreceptor degeneration (Gouras et al, 2002).

7.1.1 Ethical approval

The studies on the Abyssinian cats with hereditary cone-rod dystrophies were approved by the Swedish Animal Ethical Committee in Uppsala.

The Columbia University Animal Care & Use Committee, Health Sciences Division, approved all animal experiments performed at Columbia University. In all the animal experiments we followed the "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) and the OPRR Public Health Service Policy on the Human care and Use of Laboratory Animals.

7.1.2 Anaesthesia

The Abyssinian cats were sedated with xylazine. The anaesthesia initiated with intravenously administrated sodium pentobarbital before an intubation was performed. Thereafter, general anaesthesia was maintained by the use of isoflurane.

In the rabbit experiments, the animals were anesthetized with ketamine, 20 mg/kg and xylazine 10mg/kg, intramuscularly. The intramuscular injection was repeated in a lower dose if necessary approximately every 20 minutes.

7.1.3 Euthanasia

The cats and rabbits were euthanized with intravenous or intraperitoneal injection of sodium pentobarbital (Nembutal®).

The mice were euthanized by an intraperitoneal injection of Euthasol (40 mg/kg).

7.1.4 Mydriasis

Prior to all surgical procedures and eye examinations, the animals received topical eye drops of 2% cyclopentolate and 1% tropicamide for pupil dilatation.

7.1.5 The surgical technique

In the Abyssinian cats, the temporal lateral canthus area was prepared for surgery. The left eye underwent surgery and the right eye served as a control.

A 2-3 mm canthotomy was needed to gain surgical access to the pars plana region. The conjunctiva and Tenon's capsule were dissected to bare sclera. A scleral incision was performed with a diamond knife approximately 5-6 mm from the limbus, at pars plana. A self-retaining plano-convex lens was placed on the cornea over a cushion of hyaluronan (Healon®) for viewing the retina. A glass micropipette was introduced through the incision and directed to the central part of the fundus. The subretinal injection was performed in the central tapetal area nasal to the optic disc. After the procedure was completed, the sclerotomy was closed using two interrupted 9-0 polyglactin (Vicryl) sutures. The conjunctiva was sutured to the limbus and the canthotomy closed.

In the rabbits the conjunctiva was cut at the limbus and a scleral incision was made 2 mm from the limbus with a fifteen-degree knife (Alcon Ophthalmic). When an intravitreal infusion was needed for vitrectomy and the surgical manipulation of the retina, 6-0 polysorb sutures were pre-placed 1.5 and 3.0 mm from the limbus at the superior nasal and superior temporal quadrants before the scleral incisions were made with a 20 gauge stiletto knife. The nasal incision was used for a balanced salt solution infusion cannula stabilized to the sclera by the pre-placed sutures. The temporal incision was used for the vitrectomy instrument (Ocutome, Cooper Vision, New York). The plano-convex lens was placed on a visco-elastic cushion over the cornea to view the retina. The vitrectomy was performed at the posterior pole over the site intended for the retinectomy. A glass micropipette with a flat tip and an outer tip diameter of 50μm was introduced into the vitreous cavity to create a bleb detachment by injecting about 25 μl of a balanced salt solution through the neural retina into the subretinal space using an ultra-micro-pump II (World Precision Instruments, Sarasota, FL).

At end of surgery the temporal port was first closed with the pre-placed suture and thereafter the nasal infusion port was closed in order to be able to maintain almost normal intraocular pressure as the sclerotomies were closed. The conjunctiva was sutured to the limbus. No postoperative medication was used in the rabbits.

7.1.6 Fundus examination

The Abyssinian cats where examine by a Keeler binocular indirect ophthalmoscpe (Keeler, UK) and fundus photographs where taken by a handheld fundus camera (Kowa RC-2 camera, Kowan, Japan).

The rabbits had fundus examination by SLO (Rodenstock Instruments, Germany) using four different wavelengths: argon laser blue (488 nm) and green (514 nm), helium neon laser red (633 nm) and infrared diode laser (780 nm). Using this same instrument, fluorescein angiography (FAG) was performed by injecting 0.3 - 0.5 ml of a 10% solution of sodium fluorescein into an ear vein of the rabbit. The fundus was illuminated by blue light obtained from the argon laser and digitally photographed through a green transmitting barrier filter. Indiocyanine green (ICG) angiography was performed by injecting about 0.3 ml of ICG solution (2.5 mg/ml) into the same ear vein. The ICG system uses the infrared diode laser and a barrier filter transmitting wavelengths greater than 810 nm. The angiograms were monitored and stored by digital photography and recorded on a videocassette. The fundus was examined by biomicroscopy and in some cases fundus photography (Canon, FU-60). The digitized SLO images were transferred to an Adobe Photoshop software program for analysis and printing.

7.1.7 Histology

For histology each eye was removed within 1-3 minutes after death. The bulb was punctured with a 30 gauge needle at the temporal limbus for orientation and immersed in 2-3% phosphate buffered glutaraldehyde, pH 7.4 and kept at $+4^0$ C for 1-7 days (the mice 3-20 days). The eyes were washed in Dulbecco's phosphate buffered salt solution. The anterior segment, including the lens, was removed and the posterior eyecup examined under a surgical microscope. Target areas in the retina were located, and cut out in a small square segment with the superior temporal corner cut off for orientation. The cat, rabbit and mice retinal segments were dehydrated, embedded in either paraffin or epon, osmicated, sectioned semi-serially through the target area, stained with toluidine blue and examined by light microscopy. Selected areas in the epon block were trimmed and used for ultra-thin sectioning. These sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy.

The mice eyecups were sectioned into two pieces with a razor blade along the vertical meridian through the optic nerve head. These segments were osmicated, dehydrated using ethanol and propylene oxide and embedded in epoxy resin. Blocks were sectioned semi-serially at 1 to 2 μm in thickness, stained with 2% toluidine blue and examined by light microscopy. At selected points, mainly at the posterior pole, the block was trimmed and ultra-thin sections were cut, stained with uranyl acetate and lead citrate and examined using an electron microscope (Zeiss 100B). In two of the eyes from Rpe 65 -/- mice, aged 13 and 16 month of age, blocks were trimmed and sectioned in the inferior peripheral retina to compare this area with changes seen at the posterior pole. All sections were viewed and selected areas photographed at magnifications of 5 000 to 27 000X.

7.2 PHOTORECEPTOR TRANSPLANTATION STUDY (PAPER I)

In this paper we examined the effects of transplanting small micro-aggregates of neural retina containing undifferentiated photoreceptors of 3-5 day old normal kittens into the sub-retinal space of adult 1-3 years old Abyssinian cats with an early stage of hereditary photoreceptor degeneration. Twelve adult Abyssinian cats were the recipients of these transplants.

Both eyeballs from the kitten donor were used for each transplantation procedure. The eyeballs were dissected and the neural retina was peeled away from the RPE layer, submerged in Hank's balanced salt solution under 95% oxygen and 5% CO_2 at pH 7.4. This procedure is previously described by Gouras et al, 1994. The specimen was further processed in minimal essential media (MEM) where the neural retina was carefully dissected into small 0.5-1.0 mm aggregates and allowed to gravitate in small vials for 5 min. The supernatant was removed and the clusters of retinal micro-aggregates were sucked into a glass micropipette for the subretinal injection. The transplants were deposited subretinally in the central tapetal area, nasal to the optic disc. Usually two injections were made within a retinal bleb of about 2 disc diameter wide. A dark spot produced by some pigmented cells in the micro-aggregate could usually be seen within the bleb detachment.

Immediately after surgery the Abyssinian cats received a single dose of 0.5 mg/kg prednisolone. No daily immuno-suppression was used, even though the donor kittens were from another breed of cats. Topical cycloplegic (atropine 1%) and antibiotic (chloramphenicol) drops were given daily for a week. The eyes were examined daily the first week and thereafter every week until one month and then monthly up to six months.

For histology, the cats were sacrificed one hour, 1 and 2 weeks and 1,2,3,5 and 6 months after the transplantation. The both eyes were used for light and electron microscopy as described above.

7.3 BLEB DETACHMENTS STUDY (PAPER II)

Six adult pigmented rabbits were used in the investigation of bleb detachments. The surgical procedure started as described in 7.1.5 but no vitrectomy was performed in these sets of experiments. After the scleral incision was made, the contact lens was placed on the cornea over a cushion of hyaluronan (Healon®). A glass micropipette with a flat tip of 50 μm in diameter was attached to a silicon tube and a 1 cc syringe filled with balanced salt solution (Alcon Surgical BSS). The micropipette was inserted through the scleral incision and brought to the retinal surface just below the myelinated optic disc. The area chosen for the bleb detachment was an area easy to detect by SLO. As the micropipette gently touched the neural retina the injection started and a fine retinotomy was made and approximately 50 μl BSS was slowly injected into the subretinal space producing a circular bleb detachment of the neural retina having a diameter of about 3 mm. Care was taken to avoid touching the RPE layer. After completing the procedure, the scleral incisions were closed with 6-0 Ethilon and the conjunctiva sutured to the limbal area.

The fundus and the choroidal circulation were examined at 15 minutes to 18 hours after surgery and weekly thereafter by SLO and FAG and ICG angiography as described in 7.1.6.

The rabbits were sacrificed at 1, 2, 3, and 4 months after surgery and the eyes examined histologically described in 7.1.7.

7.4 RPE REMOVAL STUDY (PAPER III)

In this study, ten pigmented rabbits were used. The main surgical procedure is described above in 7.1.5. After the bleb detachment was achieved the neural retina was cut with a micro-scissor beginning at the injection port of the bleb detachment. The retina was displaced in order to prepare an area free from the neural retina so that a glass micropipette could be introduced under the retina. Single RPE cells or clusters of RPE cells were gently sucked up into the micropipette from Bruch's membrane with the aid of the ultra-micro-pump II (World Precision Instruments, Sarasota, FL). Sometimes a gentle rubbing on the RPE layer with a silicone-coated rod was needed to facilitate the removal of RPE cells from Bruch's membrane. Extreme care was taken to avoid any damage or hemorrhage. Bruch's membrane was easily visualized as a highly reflecting surface. The RPE débridement covered an area of 0.15-0.2 mm^2 in three animals and 0.4-0.8 mm^2 in six animals. In one rabbit, no bleb detachment or débridement was carried out, but a gentle pressure was applied on intact neural retina to investigate how gentle pressure alone could affect the choroidal circulation. The pressure was applied with a fine glass rod with a smooth, rounded tip of 1.6 mm in diameter and only a fraction of the tip was pressed on the retinal layer. An impression of a micro break of the neural layer appeared but no removal of RPE cells or hemorrhage was seen.

The rabbits were examined as described in 7.1.6 using the SLO. The choroidal circulation was monitored by FAG and ICG angiography monthly up to 1-5 months. Two of the eyes had an early examination at 15 min and another three eyes at 18-24 hours postoperatively (see table 1 in paper I).

Rabbits were sacrificed for histology at 1 (3 rabbits), 2 (2), 3 (3), 4(1) and 5(1) months after surgery and the eyes examined as described in 7.1.7.

7.5 CHOROIDAL CIRCULATION AFTER LOCAL PRESSURE (PAPER IV)

This study was performed on twelve pigmented rabbits using the same vitrectomy procedure as in paper III, described in 7.1.5. After producing a bleb detachment, the neural retina was cut open with a micro-scissor, beginning at the injection port of the bleb. The neural retina was removed with a fluted cannula creating a circular retinectomized area of about 2.5-3 mm in diameter. This area had to be large enough to avoid any possible focal area of RPE damage, caused by the jet stream effect necessary to produce the bleb detachment, because it was important to exert pressure on a completely unaffected RPE layer. A fine glass rod with a smooth, rounded, flat tip of 0.9 mm in diameter was used to exert slight, transient pressure on the retinal epithelium. This glass rod approached the RPE layer at an angle of approximately 45^0 so that only a small fraction of the tip indented the RPE and choroid. This indentation caused no hemorrhage or visible RPE loss and lasted only a fraction of a second. One pressure indentation was performed in three rabbits, two in another three, three in two and four in another rabbit. Three rabbits had only local vitrectomy and retinectomy and no pressure indentation was applied on the RPE layer.

In order to try to estimate a reasonable measurement of the magnitude of the pressure being applied to the RPE layer and choroid, the same glass rod was used to indent a thin rubber membrane observed under the surgical microscope. The indentation made in this way was compared to that produced by a Schötz tonometer. Even though the Schötz tonometer is designed to measure pressures above 1 mm Hg, estimations were made down to 0.1 mm Hg. It was assumed that equivalent pressures produced similar changes of the rubber membrane. Using these assumptions we concluded that the pressures exerted by the rod on the RPE and choroid were in the range of 1-0.1 mm Hg.

All of these rabbits were examined within 10-15 minutes after the pressure indentation or just after the retinectomy alone. The choroidal circulation was monitored by ICG angiography with the SLO as in paper I-III also described in 7.1.6. The follow-up angiograms were performed on day 1, 2 and 6 during the first week and then after a month. Two of the rabbits that underwent pressure indentation and one control rabbit were sacrificed immediately after the initial ICG angiogram to detect any histological changes that could be associated with the pressure indentation.

The other nine rabbits were sacrificed at one month after the final SLO ICG angiography examination. All eyes were prepared for histology as described in 7.1.7.

We also tried to grade the angiograms as to whether they had partially or completely recovered by using a percentile grading system, comparing the initial changes to what was found at later times after the initial insult.

7.6 BLEB DETACHMENT VERSUS RETINECTOMY; INDUCED CHEDIAK-HIGASHI-LIKE DEFECT IN RPE LAYER (PAPER V)

Fourteen rabbits were used in these experiments, in order to determine the influence of the neural retina on the morphological changes taking place in the RPE layer following bleb detachments versus retinectomy. Histological changes of the RPE cells were already observed in paper I and IV. Seven of the rabbits underwent the bleb detachment procedure when a bleb of about 3 mm in diameter was produced while seven other rabbits also had additional local retinectomy performed of the detached retina. The elevated retina, caused by the bleb detachment, was removed by a slow suction and slow cutting mode of the vitrectomy instrument (Ocutome, Cooper Vision, NY, USA) so that no RPE cells were damaged within the retinectomy area.

The fundus and the choroidal circulation in all rabbits were examined weekly during the first month and thereafter monthly by indirect ophthalmoscopy, SLO, autofluorescence, FAG and ICG angiography.

The major interest in this study was however the histological examination of the retinas. The rabbits were sacrificed at 1, 2, 3, 4 and 5 months after surgery and the eyes processed for light and electron microscopy as described 7.1.7.

7.7 AGING DEFECTS IN THE BASAL LAMINA OF THE RPE LAYER (PAPER VI)

In this study two strains of mice were studied, the RPE65-/- mutant and the wild type. The retinas from both of the eyes of eight RPE65-/- mutant mice and nine wild-

type mice of different ages from 6 weeks to 3, 6, 12-13 and 16 months of age, were examined by light and electron microscopy. Two mice from each strain were examined at the different time points.

We wanted to compare the changes in the basal lamina at different ages to what occurs in normal mice in order to determine if the changes in the mutant mice might be related to their unique genetic abnormality or was simply an unusual form of aging of the mouse RPE layer.

In two of the eyes from Rpe 65 -/- mice, aged 13 and 16 month of age, the epon blocks were trimmed and sectioned in the inferior peripheral retina to compare this area with changes seen at the posterior pole. All sections were viewed and selected areas photographed at magnifications of 5 000 to 27 000X.

8 RESULT

8.1 PHOTORECEPTOR TRANSPLANTATION STUDY (PAPER I)

Photoreceptor transplants survive for at least six months without any evidence of rejection or infection. Photoreceptors form outer segments within these micro-aggregates, although not as a monolayer but only in abnormal rosettes. The tissue integrates with the host neural retina but no evidence of synaptic formation with host retinal neurons was observed. There was evidence that the micro-aggregate size was too disruptive to the neural retina causing a large shift of the host photoreceptor layer from the RPE layer and consequently greater degeneration of these photoreceptors. There was also an indication of macrophages being attracted into the sub-retinal space by either the surgery and/or the transplant. We stated that more attention had to be given to the procedural issues involved in gaining access to the subretinal space and in minimizing potential inflammatory and/or provocative maneuvers necessary to perform surgery in the subretinal space. We consequently turned our attention to better understanding of how access to the subretinal space can be achieved. How we could optimize our ability to manipulate structures in this space and minimize any deleterious or inflammatory effects. We considered this research aim of considerable importance for the future of retinal cell transplantation as well as gene therapy.

8.2 BLEB DETACHMENTS STUDY (PAPER II)

In this series of experiments, the effects of transient bleb detachment of the neural retina on the retinal epithelial layer and the underlying choroid were studied. A bleb detachment is the traditional way to gain access to the subretinal space. Bleb detachments were found to produce several complex abnormalities, which influence both the retinal epithelial layer and the underlying choroid. First, the need to employ jet stream force to physically separate the neural retina from its attachments to the epithelium causes a local pressure injury to the epithelium and accompanying changes in the choroidal circulation, which develop at the focal target of the pressure wave. This pressure wave breaks through the neural retina and directly hits a local area in the epithelial layer where it causes a small region of damage. This produces transient blockage of flow through the adjacent choroidal vessels and long term staining of these vessels, which can be detected better by ICG than fluorescein angiography. In addition, the forced separation of the neural retina from the epithelium pulls off pieces of apical membrane of the epithelial cells throughout the detached area, a phenomenon that has also been reported by others (Lopez et al, 1995). This pressure wave also stretches the neural retina, especially rabbit retina that lacks the support of blood vessels except in a narrow region around the optic nerve head. The stretching causes the formation of folds in the neural retina after it reattaches. Reattachment usually occurs within hours after the detachment has been produced. The stretched neural retina has become too large to fit into its original position (Figure 9). These changes occur rapidly and are demonstrable mainly by angiography or post mortem histology.

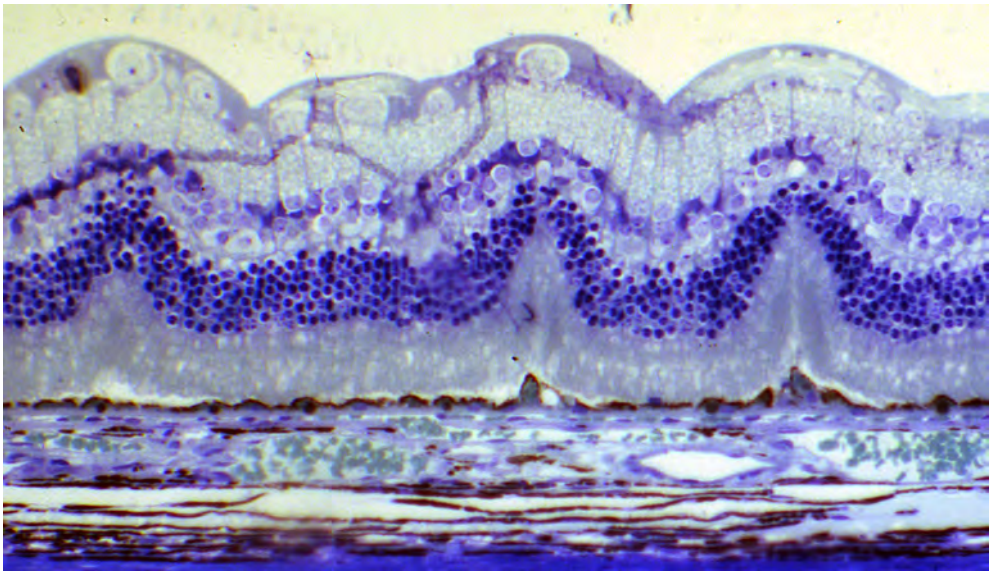

Figure 9 *LM photograph shows the foldings of reattached rabbit retina after a bleb detachment.*

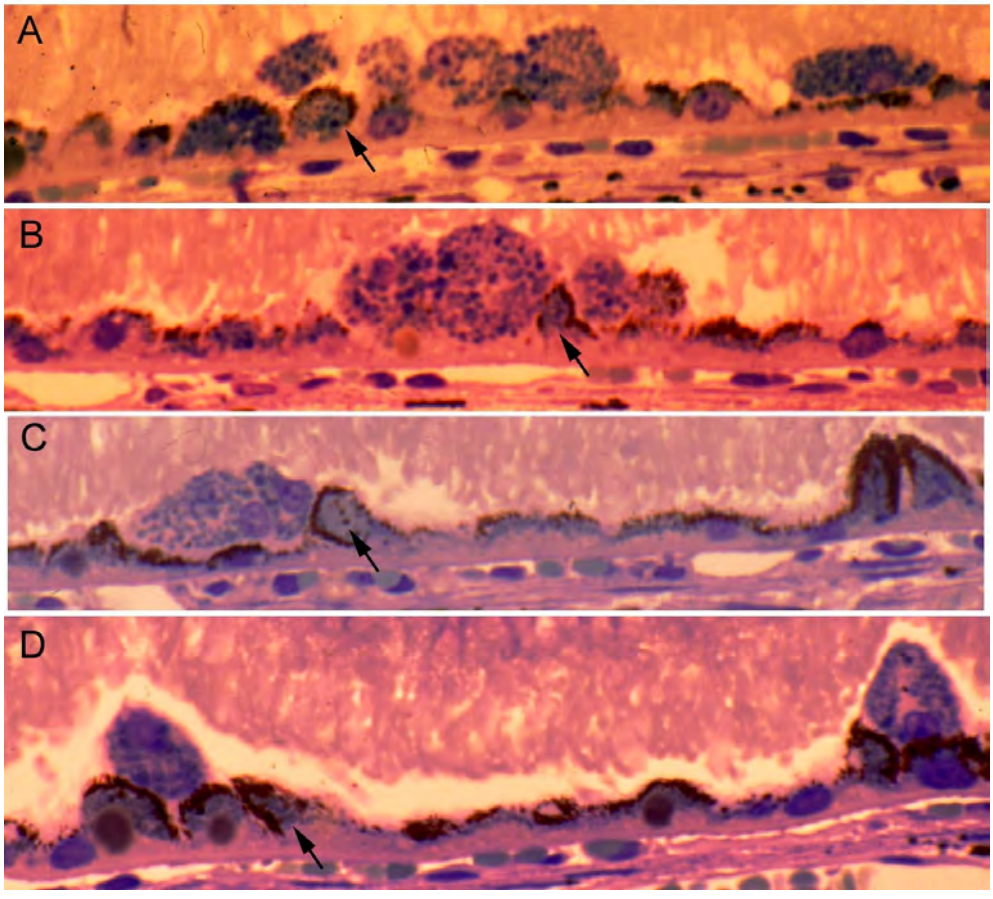

Figure 10 A-D *LM photographs show examples of proliferation, migration and transformation of RPE cells occurring within areas that had previously been detached. The arrows indicate unequivocal RPE cells migrating because of the rim of melanin pigment that marks them as such. There was never any evidence of any migrating macrophages anywhere in the retina or choroid implying that all such cells were derived from the original RPE layer.*

In addition to these rapid changes there are also unusual changes that gradually develop within the bleb detachment zone that provoke migration, mitosis and transformation of the underlying retinal epithelium seen in figure 10 A-D (Ivert et al, 2002). These changes in the retinal epithelium are especially interesting because they reveal a phenomenon that must influence the long term survival of some of the epithelial cells after such bleb detachments are performed; this could contribute to our understanding of why long term retinal epithelium transplants fail to survive in a similar position in the subretinal space.

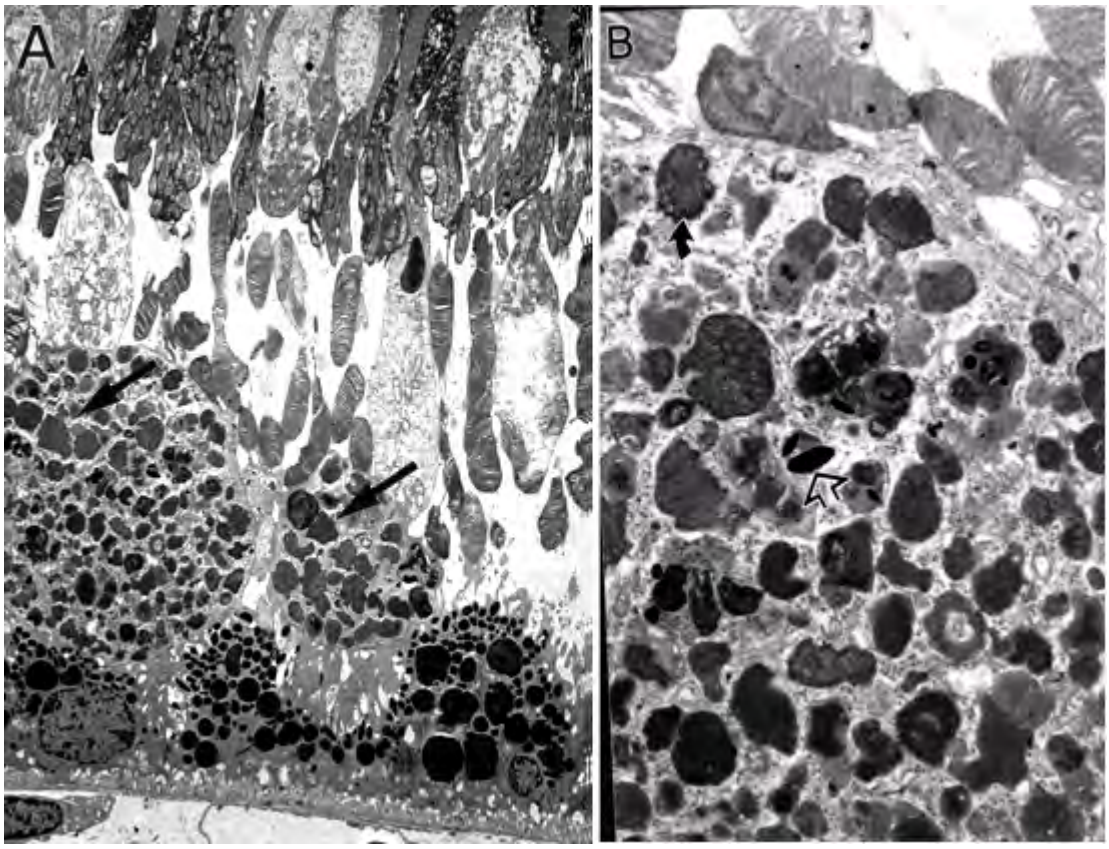

Figure 11 A*. show EM photographs of the build-up of lysosomal particles in RPE cells after a bleb detachment. The straight arrows indicate phagosomes.* ***B****. Magnified view showing the preponderance of the lysosomal debris; filled bent arrow indicates a phagosome; open arrow indicates a melanin granule.*

That a retinal detachment provokes migration and proliferation of the retinal epithelium and causes this monolayer to assume a macrophagic appearance has already been described (Machemer, 1968; Machemer & Norton, 1968; Kroll & Machemer, 1969; Machemer & Laqua, 1975; Fisher & Anderson, 1998) but the gradual and enormous accumulation of lysosomal bodies in the cytoplasm of these cells has not been detected previously. The lysosomal particles in the RPE cells are better studied in the EM magnification (figure 11 A & B). We believe the reason for this is that all previous studies involved longer term detachments where most of the outer segments had degenerated before reattachment occurred. This greatly reduces the phagocytic load on the retinal epithelial cells and therefore fails to reveal this lysosomal abnormality, which is studied more completely in Paper V.

8.3 RPE REMOVAL STUDY (PAPER III)

How removal of the epithelial layer within a bleb detachment affects the underlying choroidal circulation was examined in this paper. We found that the débridement of the RPE can lead to irreversible changes in the underlying choroid, especially if relatively large areas of retinal epithelium are removed (Ivert et al, 2003). Several methods have been used to remove the RPE layer from Bruch's membrane. One method uses calcium chelating agents, EDTA, combined with gentle brushing with a soft tip silicone catheter (Del Priore et al, 1995). Others have used a silicone brush alone (Ozaki et al, 1995) or a subretinal forceps (Valentino et al, 1995) or a bent cannula (Binder et al, 2004). Another employed a micro-jet stream to dislodge the host RPE (Parolini et al, 1995). Displaced RPE cells are then aspirated from the subretinal space (Binder et al, 2004).

A combination of suction and rubbing with a glass smooth edged micropipette was used to remove the RPE layer within the bleb detachment. Precaution was taken not to produce any damage to Bruch's membrane or hemorrhage from the choriocapillaris, which could be assessed by bio-microscopy during the surgery. In many cases, we encountered permanent alteration of the choroidal circulation, especially where relatively large areas (0.8 mm^2) were debrided. This was due to fibrosis, considered secondary to inflammation produced by the removal of the epithelial layer (figure 12).

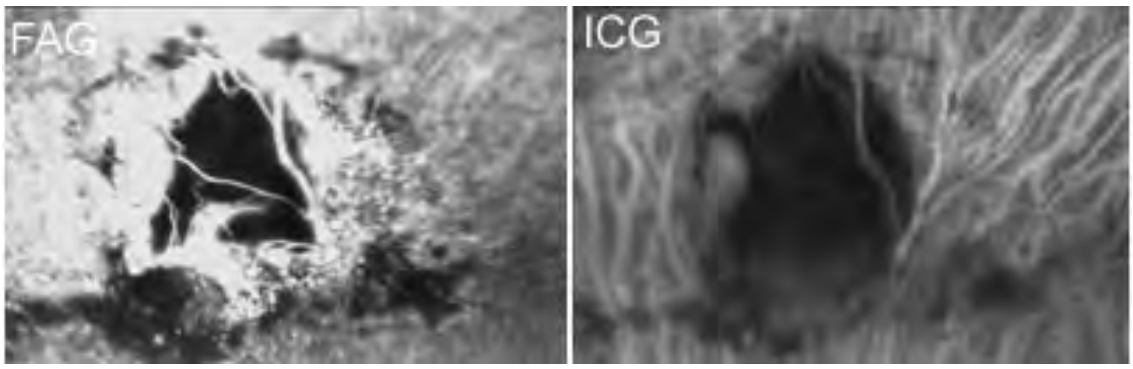

Figure 12. *SLO images of FAG and ICG angiograms taken at 4 months after removal of RPE. The different angiograms show absence of vessel perfusion in large choroidal arteries and veins and in the choriocapillary beds, which has been established by histology to be due to fibrosis in the choroid.*

A fibroblastic invasion compressed and constricted the large choroidal vessels, which led to reduced blood flow to the choriocapillaris. That removal of the retinal epithelial layer provoked the migration of fibroblasts into the choroid was also observed by Herriot & Machemer (1992) but the compression of the large choroidal vessels, which can accompany this inflammatory response, was not observed, possibly because they did not use ICG angiography. The loss in blood flow in both the large choroidal vessels and the choriocapillaris was detected by ICG angiography, which is better than FAG for evaluating the choroidal vasculature. The presence of fibroblastic infiltration around these vessels was demonstrated by post mortem histology (Ivert et al, 2003).

If the area of RPE removal was relatively small (< 0.3 mm^2), permanent changes to the large choroidal vessels were less prone to occur. All débridement of the epithelial layer was invariably accompanied by leakage of serum through the absent blood retinal barrier for weeks after the insult; this is better detected by FAG than by ICG angiography. Most leakage ceased with time although we observed leakage for as long as several months in some cases. Similar studies in feline retina also encountered leakage for at least a month after RPE débridement (Leonard et al, 1997; Wang et al, 2001). The cessation of leakage has been considered to be due to the re-growth of the retinal epithelial layer more likely by prolongation of cells rather than by cell division (Herriot & Machemer, 1992, Kroll & Machemer, 1969; Lopez et al, 1995). Another mechanism that can explain the gradual diminution in leakage from the choriocapillaris involves the hypothesis that the choroidal capillaries receive a trophic influence from the epithelial layer that maintains their integrity (Korte et al, 1984; Del Priore et al, 1995; Del Priore et al 1996; Leonard et al, 1997). By this hypothesis some of the loss of leakage after removal of the epithelial layer can be due to choriocapillary atrophy. Our experiments also provide a possible explanation for reduced choriocapillary blood flow and consequently reduced serum leakage. The constriction of the large choroidal vessels can also lead to a reduction in choriocapillary flow because these are end stage capillary beds that do not communicate directly with neighboring beds (Hayreh, 1975 & 2004, Krey, 1975). Therefore several complicating factors must be considered and possibly counteracted whenever the retinal epithelial layer is removed. It is important to minimize the surgical trauma and the area of epithelial débridement to eliminate choroidal inflammation, which can lead to fibroblastic invasion and subsequent constriction of the large choroidal vessels. This is obviously detrimental to the local neural retina as well as to the success of any retinal epithelial transplant.

8.4 CHOROIDAL CIRCULATION AFTER LOCAL PRESSURE (PAPER IV)

In the previous study (Paper III) we noticed that débridement of the RPE or only pressure on the neural retina could cause an immediate reduction of the choroidal blood flow. The histology of the debrided area revealed fibroblastic infiltration, but these findings could not explain why there was an immediate reduction in choroidal blood flow after a slight pressure on the neural retina. In order to investigate this surprising phenomenon further, we examined what occurred if only a section of neural retina was removed and a slight pressure was exerted directly on the RPE layer. By removing the neural retina, the optics was clearly improved so the choroidal circulation could easily be examined and even better by ICG angiography. As a control we also examined how retinectomy alone without any pressure being exerted on the RPE layer affected the choroidal circulation. The removing of the neural retina caused no change in choroidal blood flow. But pressure indentation of the RPE layer in the absence of neural retina led to a rapid disappearance of flow in the large choroidal vessels immediately adjacent to where the pressure had been applied. This is illustrated in Figure 13 A, C & E, ICG angiograms taken after the local pressure indentation was produced, 16 min after surgery and the same area 24 hours later (Figure 13 B, D &F).

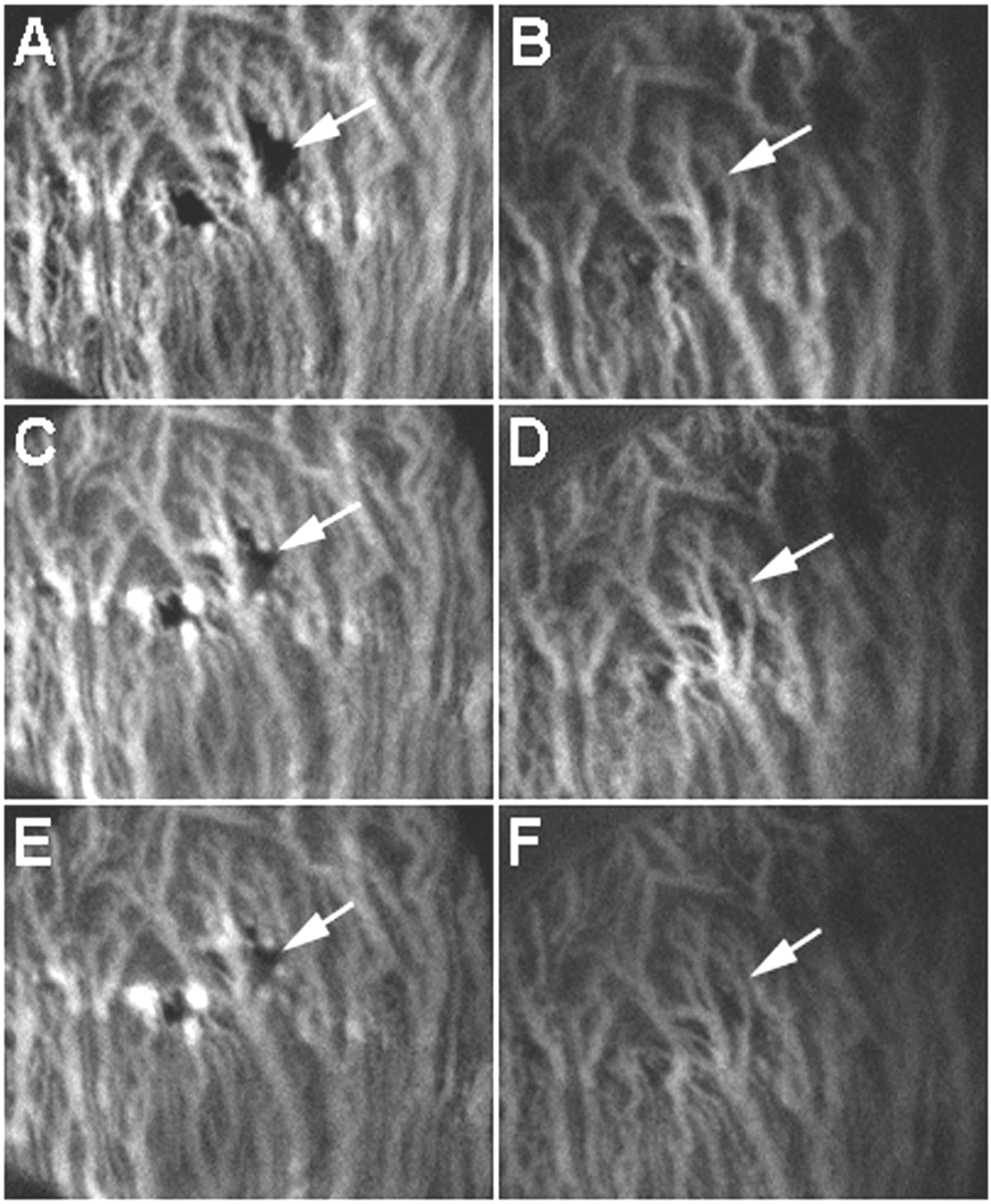

Figure 13 *show ICG angiograms taken at 1 second (**A** & **B**), 16 sec (**C** & **D**) and 38 seconds (**E** & **F**) at 16 minutes (left column) and 24 hours (right column) after surgery. Complete recovery has occurred at 24 hours.*

These areas of non-fluorescence can diminish during the time course of the angiograms, which is also illustrated in another animal (Figure 14 A-D).

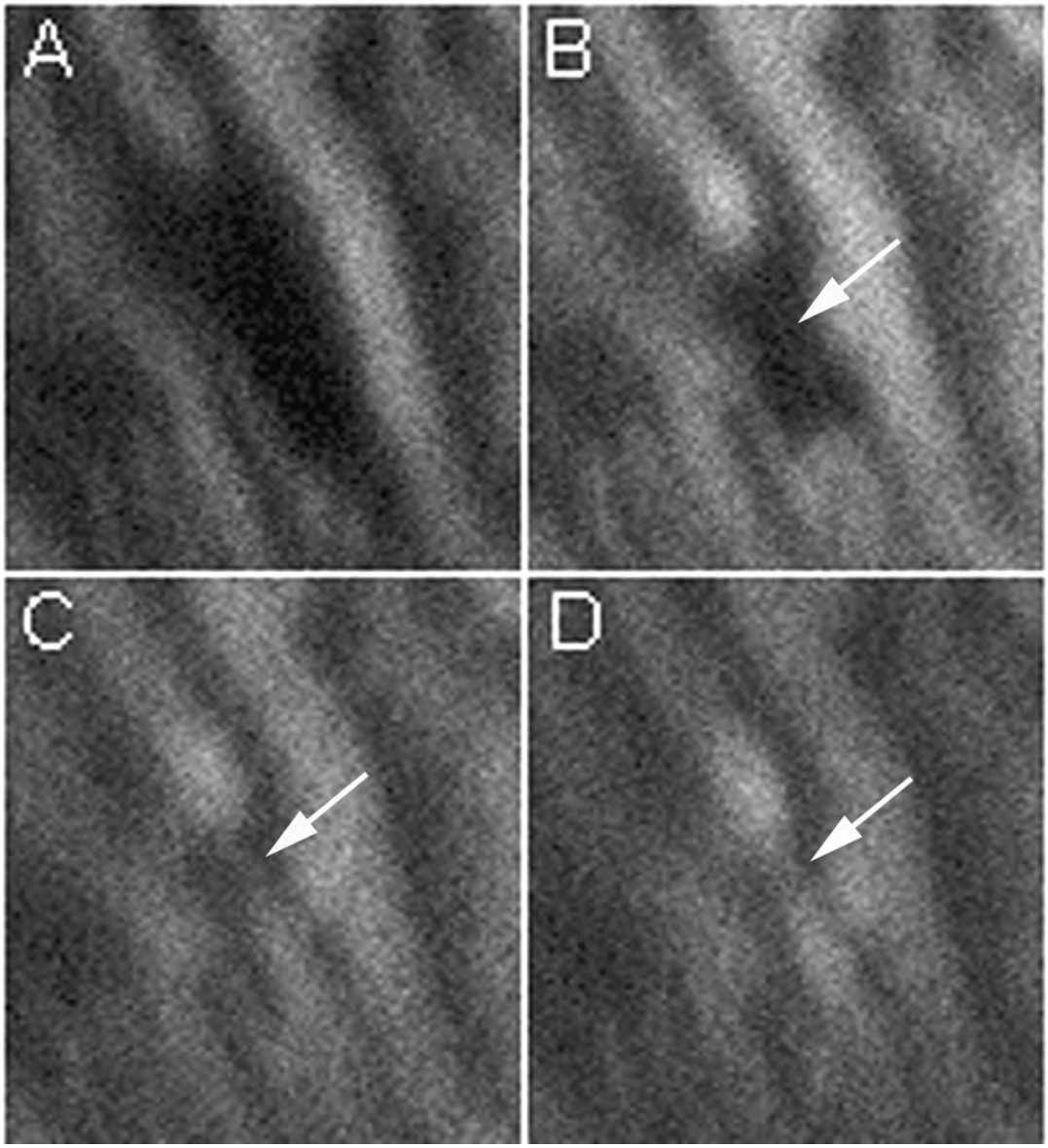

Figure 14 *show a magnified view of an ICG angiogram at 1 (**A**), 21 (**B**), 41 (**C**) and 65 (**D**) seconds, eighteen hours after the pressure indentation was applied. The angiograms show the area of non-perfusion decreases in size with time. A fine channel can be seen in B connecting the two ends of a large vein.*

All of the areas of loss of angiogram fluorescence disappear within about 24 hours as shown by comparing the percentage of recovery in different rabbits at various times after the pressure change was induced (Figure 15).

The cause of this immediate loss of flow was not clear but some of the evidence provided possible explanations. The areas of non-fluorescence gradually diminished in size during the course of the angiogram. In addition, the tips of the blocked vessels became hyper-fluorescent implying that pressure built up at these points in the vessels. An opaque material like blood or pigment that was simply blocking the fluorescence would not be expected to change during the course of the angiogram. The absence of fluorescence must be due to obstruction of ICG flow through these vessels. This obstruction could be due to a thrombus forming within the vessels or a constriction of the vessel walls produced by neuromuscular action or external pressure on the walls.

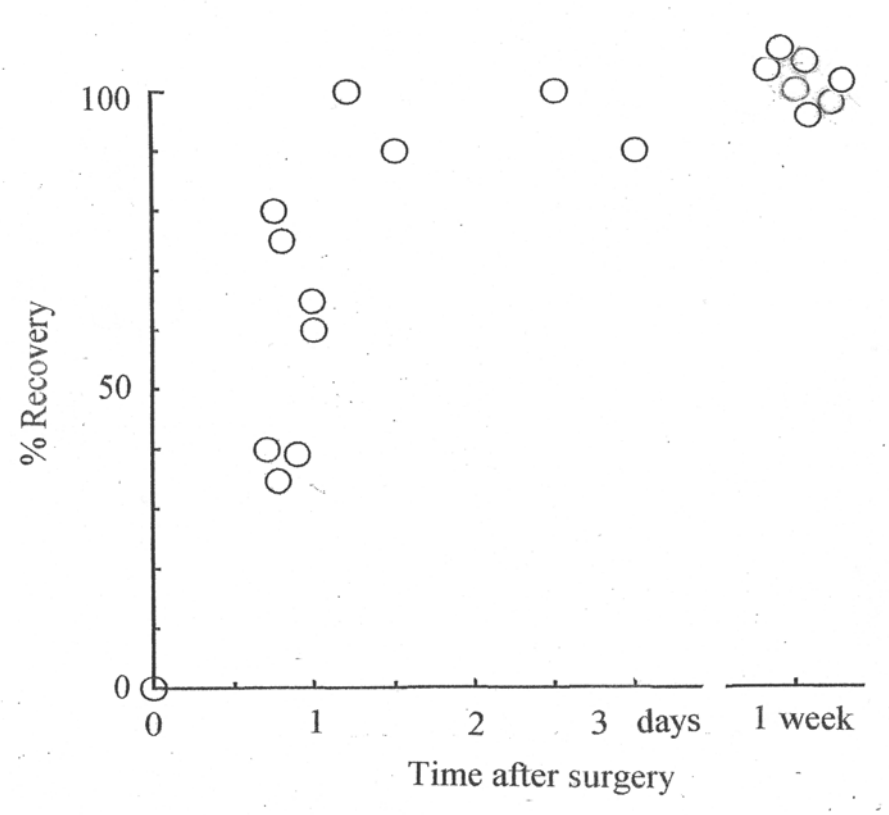

Figure 15 *shows the time course of recovery of choroidal flow after brief pressure as judged subjectively from angiograms taken at different times after the pressure change was induced.*

The areas of non-perfusion extending in a swath-like fashion across a group of vessels was difficult to reconcile with thrombus formation because it requires thrombi to form at the same position in several vessels simultaneously. A second explanation is that there is edematous pressure compressing the walls of these vessels but it is difficult to conceive of extra-cellular edema having any significant effect within a structure where diffusion must be relatively rapid. A third explanation involves a neural reflex, which causes local constriction of both arteries and veins. It is well known that the choroidal circulation has an extensive neural innervation (Flugel et al, 1994; Flugel-Koch et al 1996; Hogan et al 1971; Nilsson et al 1985; Trivino et al, 2005; Cuthbertson et al, 1997).

In order to further evaluate these three different hypotheses we turned to histology. Rabbits were sacrificed immediately after the pressure indentation had occurred, when there was maximal reduction in choroidal blood flow in the areas affected by the pressure. This confirmed that there was no extracellular material such as blood or pigment present in the choroid or the RPE layer, which could block the ICG fluorescence. What was most striking, however, was the presence of thrombotic material in the lumens of arteries and veins. This is shown in Figure 16 A & C where the choroid, which had been subjected to the pressure, contains arteries and veins in which there are concentrations of erythrocytes (16 A) and clot-like proteinaceous material (16 C) not seen elsewhere in the choroid.

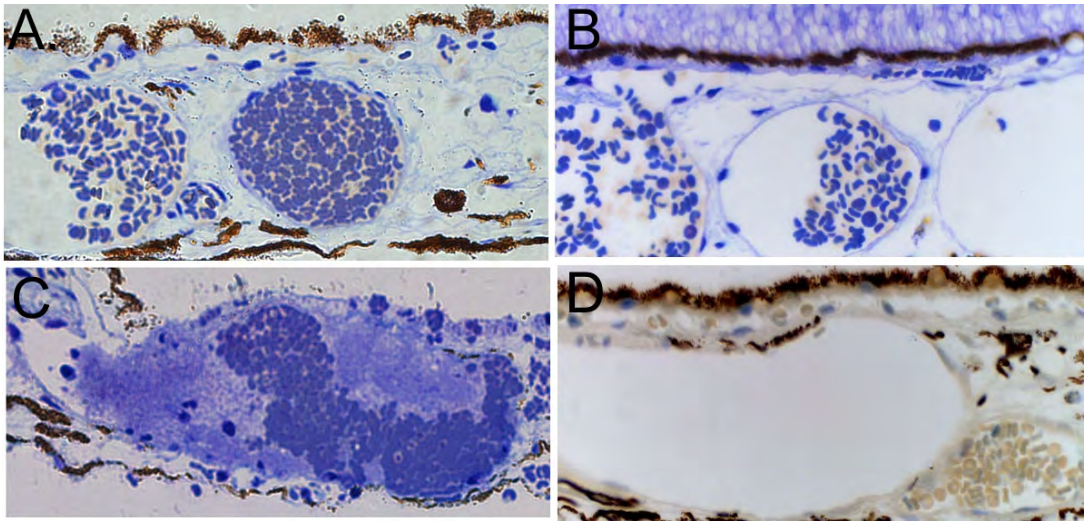

***Figure 16 A** & **C** LM photographs show areas where retinectomy and pressure have been applied. **A** shows densely packed red blood cells in a choroidal artery. **C** shows a large choroidal vein with proteinaceous, trombotic-like material. The staining of non-cellular material seen in this vein is never seen in the controls. **B** shows a control with intact neural retina and a nice monolayer of RPE cells on intact Bruch's membrane and several choriocapillaries as well as a large choroidal vein and artery without any trombotic like material. The vessels have limited numbers of blood cells within their lumen. **D** shows an area where retinectomy has been performed but no pressure being applied. The choriocapillaris and large chorodal vessels have the same appearance as the control.*

Figure 16 B, which shows neural retina and choroid in an eye where there was no retinectomy and no pressure was applied. There was no evidence of thrombotic-like material anywhere in the choroidal vessels. Also examination of the choroid in control experiments, where a local retinectomy was performed but no pressure indentation was exerted on the RPE and choroid, showed no evidence of thrombotic-like material in any of the vessel lumen (Figure 16 D). We therefore concluded that the loss of choroidal blood flow was most likely due to the formation of thrombi.

Why these thrombi are forming after the pressure indentation is unclear. The choroidal vasculature may be especially prone to thrombosis. It is also possible that neural reflexes cause constriction of these vessels and changes the flow rate, which leads to the formation of thrombi. It is well known that intravenous injection of epinephrine, that causes vasoconstriction in the venous system (Luscher et al, 1990; Haefeli et al 1993), can produce a condition similar to central serous chorioretinopathy (CSC) in an experimental animal model. There is evidence that plasminogen activator inhibitor 1, the major antifibrinolytic agent, is increased in patients with CSC, which has led to the hypothesis that the choroidal circulatory disturbance in CSC is caused by impaired fibrinolysis and a resulting thrombotic occlusion of choroidal vessels (Iijima et al, 1999). In CSC, focal areas of non-perfusion are observed with ICG angiography with edges of staining and leakage (Piccolino et al, 1995; Prunte & Flammer, 1996; Kitaya et al, 2003), which is similar to what we observe after mild pressure indentation. There is also further evidence that

a number of abnormalities, due to choroidal vessel obstruction and/or thrombosis, such as acute posterior multifocal placoid pigment epitheliopathy (APMPPE), multiple evanescent white dot syndrome (MEWDS), ampiginous and serpiginous choroidopathy are due to obstruction of flow within choroidal vessels and present clinically with areas of hypo-fluorescence on ICG angiography (Bouchenaki et al, 2002; Schneider et al, 2003). In a case of APMPPE with concurrent cerebral vasculitis, histopathology revealed thrombo-obliterative vasculitis of the medium sized arterial branches of the leptomeninges, implying that similar thrombotic changes were also occurring in the choroid (Wilson et al, 1988). Therefore there is mounting evidence that areas of non-perfusion found by ICG angiography are most often due to thrombotic involvement of choroidal vessels as we have found by histopathology in rabbits following mild pressure being applied to the RPE layer and choroid. These pressure induced thrombotic events may also underlie a condition such as Berlin edema (Berlin, 1873) where pressure to the globe induces severe retinal damage. If this were to cause thrombotic interruption of flow in the choroid that would take days to recover, damage to the retina could ensue, especially in the fovea, which depends mainly on choroidal blood flow.

The loss of ICG fluorescence not only involves the large choroidal vessels but must also involve the local choriocapillary beds because there is absence of faint fluorescence in the areas between the large vessels. This reflects a lack of choriocapillary perfusion. This loss of local capillary perfusion could be due to the fact that they are end-stage capillaries (Archer et al, 1970; Foulds et al, 1971; Hayreh, 1975 & 2004; Hirata et al, 2003; Krey, 1975; Torczynski & Tso, 1976; Yoneya & Tso, 1987; Weiter & Ernst, 1974). The non-perfused segments of the large vessels may be feeding such end-stage capillary beds.

The rapid changes in choroidal perfusion from relatively mild pressure on the RPE layer indicate that great care is needed, if RPE cell removal is a necessary step in transplantation of RPE cells (Binder et al, 2002; Van Meurs et al, 2003). Even mild pressure leads to considerable alteration in local choroidal blood flow, which takes days to recover. If the surgical manipulation is excessive, inflammation and fibroblastic invasion can occur and cause permanent obstruction of flow in the large vessels and the choriocapillaries (Ivert et al, 2003).

ICG angiography can monitor the choroidal circulation with relatively high spatial and temporal resolution. It proved to be indispensable in detecting these changes of perfusion following transient pressure to the RPE and choroid. Fluorescein angiography was less informative because of the poor visibility of the choroidal vessels. ICG provides a dynamic way to follow the choroidal circulation (Flower& Hochheimer, 1973) supplementing previous methods of assessing choroidal blood flow, such as the use of radioactive microspheres, choroidal vein cannulation (Alm & Bill,1972a & b), corrosive casting of vessels (May et al, 1996; Olver, 1990; Shimizu & Ujiie, 1978 & 1981), Doppler flowmetry and other methods (Cioffi & Alm 2001).

8.5 BLEB DETACHMENT VERSUS RETINECTOMY; INDUCED CHEDIAK-HIGASHI-LIKE DEFECT IN RPE LAYER (PAPER V)

The brief duration of our bleb detachments and the relative tolerance of the photoreceptors to the insult leave most of the outer segments intact. This presents a

continuous load of shed outer segment material for the epithelium to ingest and digest. We suggested in Paper II that this greater challenge revealed a deficiency of the transformed epithelium to digest the ingested material. In this paper we examine the electron microscopic appearance of the lysosomal material incorporated by such transformed epithelium and compare it to epithelium in which the neural retina has been removed. By the removal of the neural retina, the load of phagosomal material is completely eliminated. Figure 17 shows that there is an enormous amount of large lysosomal debris filling the entire cytoplasm of these transformed retinal epithelial cells in the bleb detachments. There are relatively few phagosomes indicating that there is not an increased ingestion of material that is causing the excessive lysosomal debris.

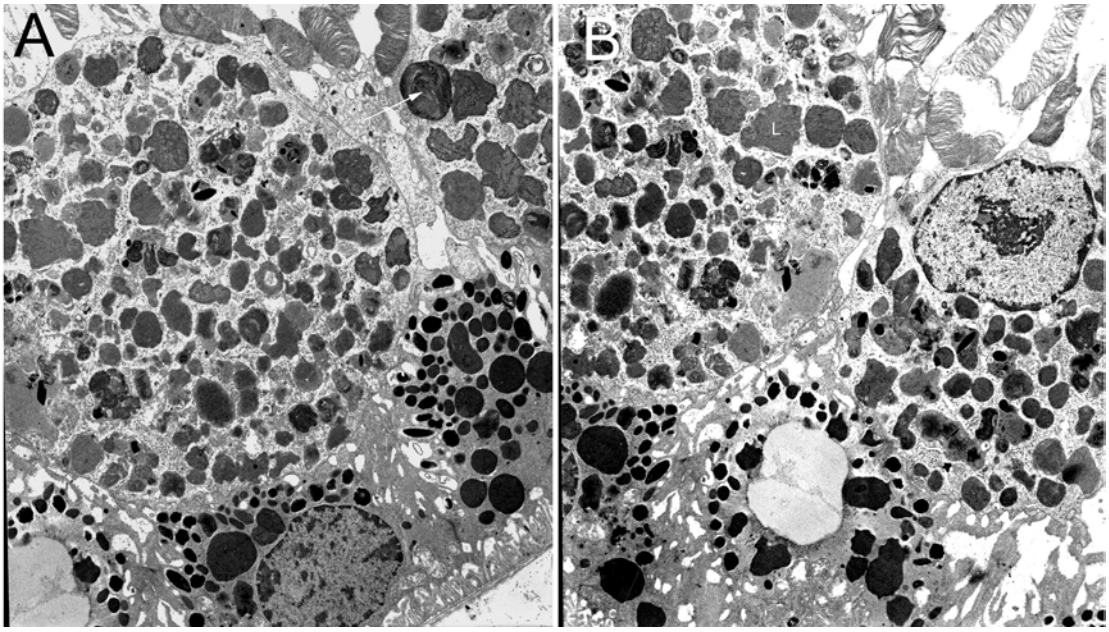

***Figure 17 A** & **B** shows EM photographs of transforming RPE after a bleb detachment. Phagosomes are less dominant (thin white arrow, figure A) than the amount of lysosomal debris (L, figure B), which is filling the entire cytoplasm of the RPE cells.*

The appearance of these cells closely resembles what is found in the Chediak-Higashi syndrome (CHS) (Robison et al, 1975; Robison & Kuwabara, 1978; Valenzuela & Morningstar, 1981; Collier et al, 1984 & 1986), a syndrome known to be due to faulty lysosomal digestion (Ward et al, 2000; Shiflett et al, 2002). By morphological analogy, this implies that the same defect is occurring in these cells, which have been transformed by the bleb detachment. This abnormality is important because faulty lysosomal digestion, as occurs in CHS, causes these epithelial cells to degenerate with age (Collier et al, 1986; Ben Ezra et al, 1980; Sayanagi et al, 2003). The transformation of these epithelial cells, leading to their gradual apoptosis and produced by the bleb detachment, could explain why long-term retinal epithelium transplants, introduced through similar bleb detachments, fail to survive with little (Zhang & Bok, 1998) or no evidence of host-graft rejection (Crafoord et al, 1999; 2000a & b; Gouras et al, 2002; Gouras, 2004).

Many of these transplanted epithelial cells do in fact look like these transformed epithelial cells (Crafoord et al, 1999; 2000 a & b) but they have been called macrophages rather than transformed epithelium. In our bleb detachments, none of these transformed epithelial cells could have been macrophages that migrated into the subretinal space. The reason for this is that all stages of transformation could be seen in the retinal epithelium, that no such transformed cells were found elsewhere in the retina or choroid and that these transformed cells were only present at the folds in the neural retina where the photoreceptors and neural retina are displaced from the epithelial layer. Therefore we conclude that only retinal epithelial cells comprise all of these transformed cells, which indeed resemble macrophages because of their excessive amounts of lysosomal debris.

This leads to the question of what is causing these otherwise quiescent retinal epithelial cells to migrate, divide and accumulate excessive lysosomal debris. An important clue to this process is provided by the fact that the transformation is triggered by the folds in the neural retina. It would seem that the displacement of the neural retina from the epithelium triggers in some way the transformation, perhaps by release of a factor that prevents RPE from transforming. In order to test this, we performed local retinectomies, which completely eliminates any factor that can influence the epithelial layer. Figure 18 shows that when this is done, there is a most extraordinary transformation of the RPE layer, much greater than anything we find at the folds in the neural retina. The RPE layer migrates and forms multiple layers of epithelia. But there is no accumulation of lysosomal debris (Figure 19), undoubtedly because there are no outer segments. This suggests that the neural retina transmits a continuous signal to the RPE layer that keeps this layer quiescent. If the neural retina is lost or even displaced from the RPE layer, the transformation of this layer begins. This is of course completely consistent with the research on the long-term effects of retinal detachments (Fisher & Anderson, 1998). Such retinal detachments also lead to migration and transformation of the epithelial layer and in some cases to the migration of these cells into the vitreal cavity to produce proliferative vitreoretinopathy (PVR) (Machemer et al, 1978). It is interesting; however, that in such long term detachment studies the enormous buildup of lysosomal material by these transforming epithelial cells has not been as noticeable. The reason seems to be that in bleb detachments, there is little to no loss of outer segments and therefore a continuous load of phagosomal material is being presented to these cells, which reveals their lysosomal digestion defect. In long-term detachments, there is considerable loss of outer segments and therefore the phagosomal load is less.

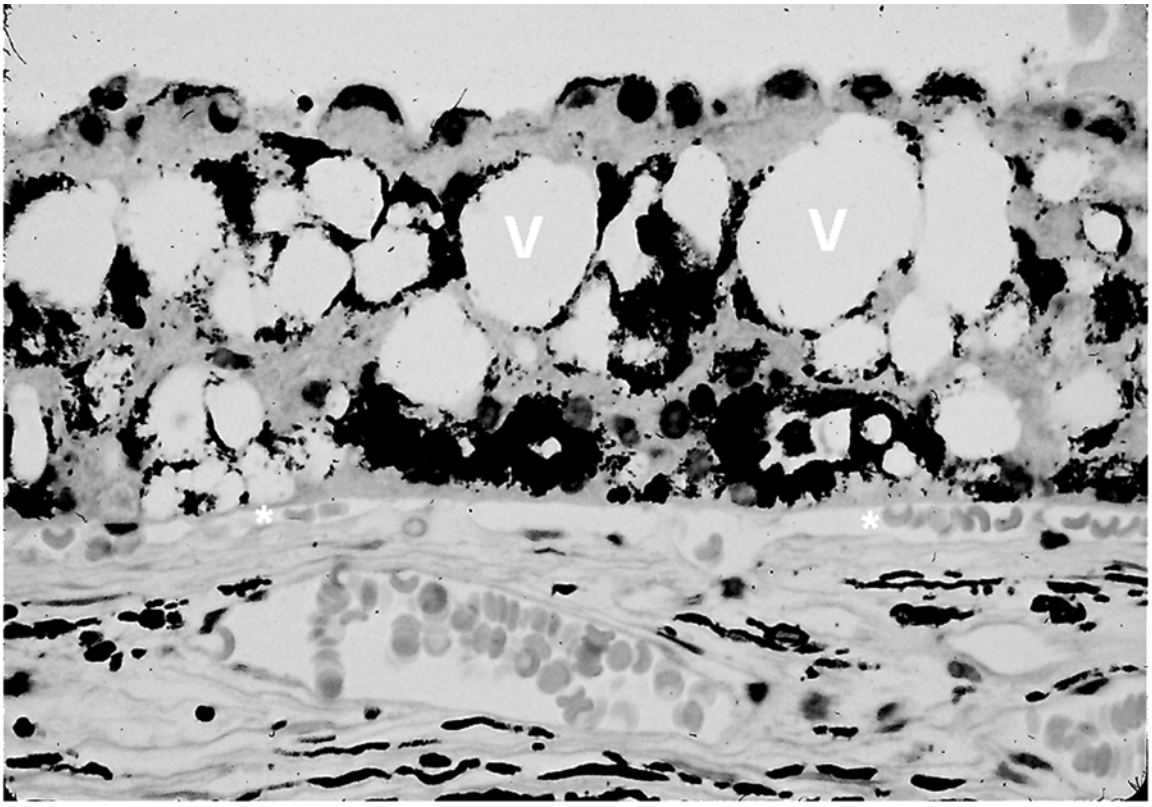

***Figure 18**. LM photograph shows the effects of a local retinectomy on the RPE layer at 4 months after surgery at the light microscopic level. The monolayer has now become multilayered with numerous cystic structures formed (V) by small groups of RPE cells. The removal of the neural retina has produced an enormous proliferation and migration of RPE cells. Intact choriocapillaries are seen below the RPE (asterix)*

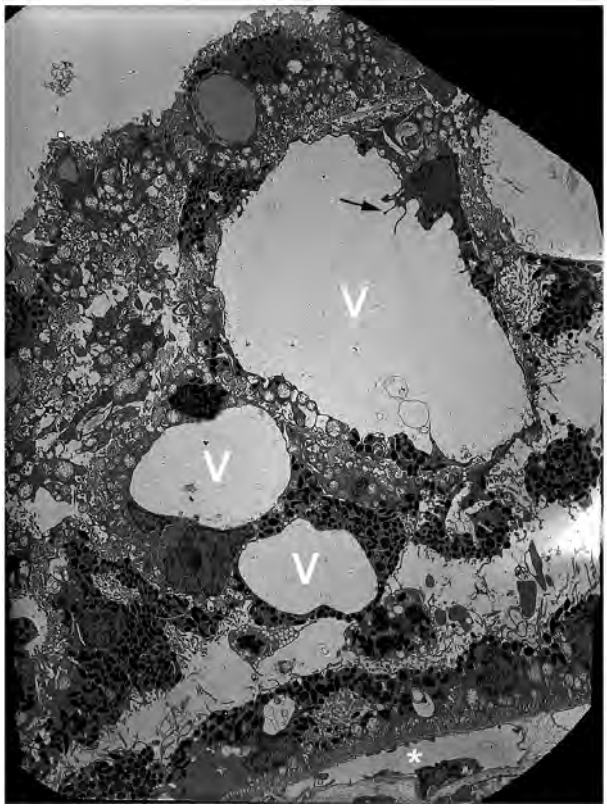

***Figure 19** shows these changes at the EM level. Large vacuoles are seen (V) within the RPE cells. It is noteworthy that there is no accumulation of lysosomal material within the cytoplasm of these RPE cells, which must be due to the absence of effete outer segments from photoreceptors*

8.6 AGING DEFECTS IN THE BASAL LAMINA OF THE RPE LAYER (PAPER VI).

The changes observed at the EM level in the ageing RPE -/- mutant mice involved an extension of the basal lamina of the RPE layer deep into the cytoplasm of the basal side of the cell. These extensions formed channels separating segments of the basal cytoplasm from the body of the cell. This increased considerably with age. A similar pattern was evident in wild type mice but was minimal in comparison to the RPE 65 -/-mutant mice. The reason for the possible difference could be the large amounts of retinyl esters that accumulate in the mutant RPE cell because of the failure of retinol isomerization (Ivert et al, 2005). The phenomenon is interesting because similar changes in the ageing RPE of human subjects are thought to lead to the formation of drusen, (Burns & Feeney-Burns, 1980; Ishibashi et al, 1986), a precursor of age related macular degeneration in man.

9 DISCUSSION

In the first experiment (Paper I) we determined how well micro-aggregates of neural retina containing photoreceptors can be placed in the subretinal space of degenerative feline retina and how well these aggregates survived. We found that the transplantation could be performed and survival of the cells could be followed for at least 6 months. But several problems arose that were not the optimal for using this approach to treat photoreceptor degeneration. First there was no evidence of any obvious neural communication between the transplant and host neurons. The photoreceptors were found in abnormal rosettes in which macrophages were found. The neural retina of the host was displaced form the retinal epithelial layer resulting in more photoreceptor degeneration. The exposure and effects of producing a bleb detachment necessary for these transplantation experiments was too traumatic. We then turned our attention to examining the effects of such bleb detachments and trying to understand how this technique might be improved.

The subsequent experiments (Papers II-V) reveal how interdependent the neural retina, the retinal epithelial layer and choroid are and how they interact and influence one another. In the first set of experiments (Paper II), we determined the effects of producing a transient bleb detachment of the neural retina using a relatively standard technique of injecting a small amount of a balanced salt solution into the subretinal space through a small hole produced in the neural retina by jet stream force. This technique allows access to the retinal epithelium and the outer segments of the photoreceptors, which can be useful in transplantation or gene therapy. It was shown that such a procedure has both short and long term effects on all of these structures. The short term effects involved the pressure break in the neural retina, damage to the epithelium layer and the choroid at the focal point of the jet stream force needed to separate the neural retina from the epithelial layer, and the shearing of the apical plasma membranes of the epithelial layer apparently tightly bound to the matrix material embedding the outer segments of the photoreceptors. These are all abnormalities that would be better avoided in a more ideal method of gaining accessibility to the subretinal space.

Even more intriguing, however, were the long term and somewhat unexpected changes that occurred after the production of such a bleb detachment, namely a rapid transformation of the retinal epithelial layer. The trigger for this change appears to be the loss of a signal that must come from the neural retina, which prompts this otherwise quiescent, non-replicating monolayer to migrate, replicate and drastically change its cellular metabolism. This phenomenon is clearly related to the affects of retinal detachment which had been studied intensively over many decades (Machemer, 1968; Machemer & Norton, 1968; Kroll & Machemer, 1969; Fisher & Anderson, 1998) but the bleb detachment provides a unique insight into this phenomenon that was not evident from any of the previous studies of the consequences of retinal detachment. The reason for this is that all of these previous studies of retinal detachments involved long term detachments in which there was considerable degeneration of the photoreceptor layer and some of the inner retina. Bleb detachments are very short term with reattachment occurring within hours. This leads to a virtually normal neural retina being re-apposed to the epithelial layer. This reveals several important new facets concerning the problem of retinal detachment. First, the pressure separation stretches

the neural retina, perhaps more in the rabbit because of the absence of any significant vasculature. This creates folds in the reattached retina, which are precisely the regions that trigger the transformation of the epithelial layer. Only in these areas do you find the transformation of the retinal epithelium. These migrating, macrophagic-like transformed cells are seen nowhere else in the retina or choroid. This eliminates the possibility that these transformed cells are wandering macrophages that invade the subretinal space, a hypothesis often considered as a possible source of the invading cells that contribute to proliferative vitreo-retinopathy (PVR) following retinal detachments. The second point of importance is that the transforming cells accumulate enormous amounts of lysosomal debris. The logical explanation for this abnormality is that these epithelial cells have lost their ability to digest phagosomes obtained from the adjacent photoreceptors. This abnormality was never noticed in previous long-term detachment studies because the outer segments had all degenerated eliminating the phagosomal load that is presented to the epithelial cells in these folds. The hypothesis that there is faulty lysosomal digestion in these transformed epithelial cells is supported by the striking similarity of these cells to those found in Chediak-Higashi disease, an abnormality known to be due to faulty lysosomal digestion (Ward et al, 2000; Shiflett et al, 2002). In this syndrome the retinal epithelial cells with this faulty accumulation of lysosomal debris go on to apoptosis and a retinal degeneration. This apoptosis may explain why transplanted retinal epithelium, which often are found with such lysosomal debris and are located within areas of previously detached retina also degenerate with time without any evidence of host graft rejection.

An intriguing question is why this transformation starts in the folds of the neural retina. The most reasonable explanation is that the separation of the neural retina from the adjacent epithelium removes a regulatory factor that is preventing the epithelium from transforming. It must be the same factor responsible for epithelial cell migration and transformation in long-term detachments (Fisher & Anderson, 1998). Therefore the neural retina must produce and release some factor that controls gene expression in the epithelial layer. It must almost depend upon membrane to membrane contact for this factor to be effective because this relatively slight separation of the photoreceptor layer from the epithelium is significant to trigger a local transformation of the epithelium. We obtained further evidence for this hypothesis by removing a segment of neural retina (Paper V), which leads to massive migration and replication of the epithelial layer. But there was no accumulation of lysosomal debris in the epithelial cells within this segment undoubtedly because there are no photoreceptor outer segments. This must be an inhibitory factor produced by the neural retina that controls the stability of the epithelial layer because the transformation of the epithelial is confined precisely to the segment that underwent the retinectomy. If there were a stimulatory factor/-s being released by the damaged retina it would influence the perimeter of this segment altering the behavior of the retinal epithelial layer external to this segment. Because the epithelial transformation occurs exclusively within this segment implies that a local inhibitory factor produced by the neural retina, which prevents the transformation of the retinal epithelium, has been lost. It would be extremely interesting to know what the molecular basis of the epithelial transformation is. What is the molecule or molecules produced by the photoreceptors and perhaps other elements in the neural retina that influence the behavior and consequently gene expression of the RPE? It would have considerable relevance to possibly controlling PVR development.

Another interesting consideration in relation to this transformation of the epithelial layer produced by bleb detachments is the fact that drusen, as illustrated in our Figure 6, are essentially detachments of the retinal epithelial layer from Bruch's membrane which also displaces the neural retina from the choroidal circulation, as a bleb detachment does. But this separation does not provoke any transformation of the retinal epithelium as it does when the neural retina is separated from the epithelium. Therefore it is not any separation of the neural retina from the choroid and/or Bruch's membrane that provokes the transformation of the epithelium but only separation of the epithelium from the neural retina that does.

We also examined how the removal of the RPE layer influences the choroid (Paper III). Relatively small areas of RPE debridement caused no permanent alteration of the choroid but larger areas (0.8 mm^2) produced a permanent change in choroidal blood flow. This was due to a fibroblastic invasion and fibrosis around the large choroidal vessels, which compressed their lumens and restricted flow in the end stage choriocapillary beds. This could be monitored by ICG angiography and then confirmed by postmortem histology. One of the theories for choriocapillary atrophy after RPE removal is that a trophic factor released by the epithelium is responsible for the viability of the endothelium (Korte et al, 1984). Our evidence that the manipulations needed to remove the RPE layer can also produce an inflammatory-like response in the choroid that attracts fibroblasts, which cause fibrosis and compression of the feeder vessels to these choriocapillary beds remains as another possibility. What was most surprising was the serendipitous finding that pressure alone on the RPE layer led to an almost instantaneous blockage of flow in both large choroidal vessels and the adjacent choriocapillaris, which gradually disappeared over many hours. A number of experiments were needed to understand the cause of this local blockage of choroidal flow (Paper IV). ICG angiography provided clues that allowed us to conclude that this was a blockage of flow and not an accumulation of some opaque material, such as blood, that was preventing the light from reaching these vessels. The major reason for eliminating this hypothesis was that the blockage gradually diminished during the time course of the angiogram contemporaneously with the appearance of staining at the blocked tips of the vessels. This evidence allowed us to conclude that something was obstructing flow in these vessels, both arteries and veins. This could be due to an external force, such as edema, or an internal obstruction such as thrombi or neurally induced constriction. Histology from specimens obtained immediately after the angiography showed blockage, revealed thrombotic-like material in the local choroidal arteries and veins. This has led to the conclusion that thrombi were responsible factor for the prolonged but reversible interruption of flow. It suggests that thrombi can form rapidly in large choroidal vessels from relatively gentle pressure exerted on this structure. There is mounting evidence that a number of clinical problems associated with choroidal dysfunction such as CSC, MEWDS and APMPPE are due to thrombotic events (Bouchenaki et al, 2002; Schneider et al, 2003). The well known functional deficits produced by ocular trauma, such as Berlin edema, may also be the result of pressure induced choroidal thrombosis. The tendency for thrombi to form in choroidal vessels may be related to the extensive neural network that must regulate choroidal blood flow (Trivino et al, 2005). Pressure may induce constriction of these vessels, which in turn leads to the formation of thrombi. The rabbit model of pressure induced blockage of choroidal vessels may provide a way to experimentally examine the causes

of choriodal thrombosis, especially in conjunction with in vivo methods to monitor choroidal blood flow as offered by ICG angiography.

The final paper in this series (Paper VI) investigated a phenomenon that was initially observed in the aging RPE 65 -/- mutant mouse retinal epithelium, which was being studied by the laboratory of Professor Gouras in transplantation experiments. There was an enormous in-growth of the basal lamina of the retinal epithelium that was forming multiple islands of cytoplasm at the basal surface of the epithelium. The question was whether this was a normal response of aging of the retinal epithelium or whether it was unique to this mutant strain. It turned out that the phenomenon also occurs in wild type mice but is exaggerated in the RPE 65 mutant. This could be related to the high quantities of retinyl esters that abnormally accumulate in the mutant retinal epithelium.

The observation is very interesting because one of the possible causes of drusen, a risk factor for age-related macular degeneration in man appears to be caused in part or possibly completely by the degeneration of the basal cytoplasm of senescent retinal epithelium in man and monkeys (Burns & Feeney-Burns, 1980; Ishibashi et al, 1986), especially in the macula. This is a problem we are currently studying in a monkey model of age related macular degeneration.

10 CONCLUSIONS AND FUTURE DIRECTIONS

The results of this research reveal a number of problems that occur with the creation of a simple bleb detachment, as currently carried out. First of all, the hydrostatic force used to detach the neural retina is destructive. It produces focal damage to the neural retina as well as the local RPE layer and choroid. In addition, the separation of the photoreceptors from the apical membrane of the RPE layer causes apical membrane rupture. These changes occur immediately after the procedure is initiated. It would be useful to devise a way of separating these two surfaces more atraumatically and physiologically, perhaps by using a slower injection of the detaching solution and/or by the use of a finer glass pipette that would minimize damage to the neural retina. In addition, consideration should be given to the forces, ionic and molecular, that bind the apical membrane of the RPE to either the matrix material surrounding the outer segments and/or the membrane of these outer segments. The use of solutions containing low calcium and/or low magnesium or solutions that selectively buffer these ions should be tested. In addition pH changes that could alter the electrostatic and molecular forces that influence the adhesion of these membranes could be incorporated into the detaching solution.

In addition, even more significant long-term changes cause a progressive transformation of the RPE layer leading to cell migration, proliferation and more significantly to a major defect in the degradation of ingested material. The latter can lead to apoptosis of these RPE cells endangering the fate of either the host RPE or transplanted RPE. Understanding the factors that induce this transformation is important. A clue to this problem has been obtained from observations that this phenomenon is exacerbated at folds in the neural retina. At such folds there is more separation of the neural retina, including the photoreceptors, from the RPE layer. This suggests that a type of mini-detachment provokes the RPE layer transformation. We believe that it is the mildness of this detachment that has revealed the lysosomal defect that occurs in these transforming RPE cells. Some factor coming from the neural retina must be normally preventing this transformation of the RPE layer. Because just a mild separation of the neural retina from the RPE layer as occurs at these folds must exert its influence very locally, perhaps through direct membrane to membrane contact between the photoreceptors and the apical membrane of the RPE cells. But it cannot be entirely the result of close membrane to membrane contact because the effects on the RPE layer are greatly increased by removing the neural retina entirely. Therefore even at a distance the neural retina continues to exert some influence on the status of the RPE layer. The absence or removal of this factor must be a triggering event leading to the RPE transformation. It would be valuable to examine changes in gene expression in both the photoreceptors and the RPE as this transformation progresses in order to better understand its molecular basis. Because this transformation leads to lysosomal dysfunction and consequently apoptosis, as occurs in the Chediak-Higashi syndrome may explain why all RPE transplants, including autologous ones, do not survive for long periods of time despite the absence of any host/graft rejection.

Removal of the RPE layer, which is a logical way to begin any attempt to reconstruct an RPE layer by transplantation, is also froth with many complications. Mere pressure on the RPE leads to rapid alteration in choroidal blood flow, which takes

many hours to recover. The cause of this change in choroidal blood flow appears to be due to thromboses induced in the choroidal vessels. The reason why choroidal vessels are more prone to thrombosis is not well understood but appears to be at the root of a number of other clinical abnormalities such as CSC, APMPPE and MEWDS. It may also be responsible for Berlin edema. The pressure indentation paradigm coupled with ICG SLO angiography in rabbits may serve as an animal model for studying the mechanisms of choroidal vessel thrombosis. For example instead of pressure neural transmitters or polarizing agents could be applied to the RPE layer while the vasculature is observed in order to determine if a neurospastic factor causes an initial constriction of large choroidal vessels and subsequently to thrombosis. ICG angiography and surgical manipulation could allow a means of analyzing how neural factors could affect choroidal blood flow.

More extensive manipulation of the RPE layer in order to remove it completely can lead to permanent alteration in the circulation of large choroidal vessels. This can jeopardize the entire transplant strategy. One explanation of this phenomenon is that the removal of the RPE leads to secondary choriocapillary atrophy due to the loss of a trophic factor being released by the RPE. This is thought to be prevented by the spread of neighboring RPE cells to cover the defect. If the defect is too large then the choriocapillaries not covered by RPE would atrophy. Our results indicate another possibility. The blocked choroidal blood flow in large choroidal vessels coupled with the restricted circulation of end-stage choriocapillary beds could also lead to an absence of capillary perfusion. This complication occurs more frequently when relatively large areas of RPE are removed. For success in any RPE transplantation therapy this complication should be avoided either by minimizing the area within which the RPE is removed or by improving the technique for debriding RPE from Bruch's membrane.

Our research has demonstrated that surgical manipulation of the structures bordering the subretinal space can reveal many phenomena about their interactions and mutual dependence when coupled with sophisticated monitors of structure and function such as SLO, ICG and FAG angiography coupled with light and electron microscopy.

11 SVENSK SAMMANFATTNING

Det retinala pigmentepitelet är ett enskiktat epitel som normalt ej återskapas. Vid olika degenerativa sjukdomar eller pga åldersrelaterade degenerativa förändringar kan man se destruktion av detta cellager. Det retinala pigmentepitelet spelar en väsentlig roll i samspelet mellan fotoreceptorerna, det subretinala rummet, basalmembranet och den choroidala mikro- och makrocirkulationen. Epitelcellerna tar upp avstötta, åldrade och förbrukade lameller från fotoreceptorernas yttersegment. De stabiliserar och reglerar vätskeflödet mellan choriokapillaris och neuroretina. Vidare är pigmentepitelcellerna en del av basalmembranet som kallas Bruch's membran.

Avsikten med detta arbete har varit att studera hur mikrokirurgisk manipulation av neuroretina och pigmentepitel påverkar de olika cellskikten; det choroidala flödet, pigmentepitellagret i sig och neuroretina, för att få en bättre insikt i de möjligheter och svårigheter som uppstår vid celltransplantation eller genterapi. Dessutom har åldrandet av basalmembranet studerats på friska och sjuka möss.

Genom att skapa en liten lokal blåsformad näthinneavlossning kan tillträde till det subretinala rummet beredas för olika former av terapier, såsom celltransplantation eller försök med att transplantera genmodifierade celler. Tyvärr är detta en inte helt komplikationsfri väg, ty då spatiet mellan neuroretina och pigmentepitellagret skapas, stimuleras en rad reaktioner hos pigmentepitelet, neuroretina och choroidea.

Själva injektionshastigheten, vid skapandet av den retinala blåsan, orsakar skada hos de pigmentepitelceller som träffas av vätskestrålen vid injektionen. Det finns då en stor risk att vissa av dessa celler lossar från basalmembranet. Vidare kan pigmentepitelcellernas apikala utskott, vilka omsluter fotoreceptorernas yttersegment, likaledes skadas då neuroretina lyfts upp för att skapa det subretinala rummet.

Den näthinneavlossning som skapas på detta sätt orsakar, åtminstone på kanin som saknar retinala kärl, en töjning av neuroretina. När den retinala blåsan minskar och neuroretina skall återta sin ursprungliga plats mot pigmentepitelet, är neuroretina för stor. Det kommer då att bildas retinala veck mot pigmentepitelet. De pigmentepitelceller som finns i regionerna av de neuroretinala vecken, kommer att få en större mängd avstötta yttersegment att ta hand om. Pigmentepitelcellerna kommer pga detta att stimuleras till en ökande fagocytos vilket tycks leda till att cellerna successivt förändras i sitt beteende och utseende.

Vid transplantation av neonatala fotoreceptorer till det subretinala rummet ses cellöverlevnad med utväxt av yttersegment, men fotoreceptorerna utvecklas ej i ett cellager utan man ser olikstora anomala rosettformationer.

När pigmentepitelceller tas bort kirurgiskt från basalmembranet över stora eller små områden fås initialt en cirkulationsstörning av såväl choriokapillaris som större choroidala kärl. En tilltagande fibros av choroidea uppstår med kvarstående cirkulationspåverkan. Om enbart en mindre mängd pigmentepitelceller tas bort från ett begränsat område, kan den initiala cirkulationsstörningen vara reversibel. Vid dessa studier observerades även att den choroidala cirkulationen kan påverkas redan vid ett minimalt tryck mot neuroretina eller pigmentepitelskiktet. Vid ett knappt mätbart tryck, under bråkdelar av en sekund, fås en ögonblicklig cirkulationspåverkan. Denna cirkulationssvikt kan eventuellt orsakas av en kärlsammandragning med eller utan trombos bildning. SLO ICG angiografi har varit ett utmärkt hjälpmedel att in vivo kunna följa förändringar i den choroidala cirkulationen.

Då belastningen av förbrukade yttersegment från fotoreceptorerna minimeras genom att neuroretina avlägsnas, fås istället en kraftig proliferation av pigmentepitelceller. Cellernas volym ökar drastiskt och enorma vätskefyllda blåsor utvecklas intracellulärt. Denna proliferation av transformerade pigmentepitelceller indikerar att neuroretina avger signaler till pigmentepitelet som stabiliserar detta cellager under normala förhållanden, men vid avsaknad av neuroretina sker en kraftig cellförändring.

Degenerativa förändringar av basalmembranet hos unga och åldrande möss, friska eller genetiskt defekta RPE-/- knockout möss har närmare studerats med hjälp av elektron microskopi. De histologiska förändringar som observerades har stora likheter med de förändringar som ses vid åldersrelaterad makula degeneration (AMD). Vidare studier i detta område bör fortsättas för att ge ytterligare upplysningar om åldrandet.

12 ACKNOWLEDGEMENTS

Welcome to the most read pages of a thesis (Brundin, 1988).

On these pages I will make a serious attempt to express my gratitude to all of them who deserves it, without forgetting anyone. This will probably be much more difficult than writing the pages that you just have read (or have you?)

First of all I wish to express my sincere gratitude to Professor *Jan Ygge* without whose friendliness and positive attitude this book would never have reached reality. His invaluable support and lots of late e-mails, long after midnight, made it possible.

Professor Emeritus *Peep Algvere* for his initial inspiration and good sense of humor.

Professor *Peter Gouras*, my unofficial supervisor and co-author, for letting me work in his laboratory, for all the great ideas and stimulating discussions about my findings and his endless enthusiasm even though it looked hopeless at times and his sensible opinion about what is a "waste of time".

My co-author Professor *Kristina Narfström* for her enthusiasm and expertise about cats as patients although I turned out to favor the more docile rabbit…

Associate Professor *Staffan Stenkula*, for being my teacher in vitreo-retinal surgery, for sharing so many fantastic experiences in the OR and for all the fun and challenges we encountered with Avi Grinbladt, producing the first films ever of vitreo-retinal surgery and in "writing history".

My co-author *Hild Kjeldbye*, her fantastic Norwegian friendship, her invaluable support and efforts at the ultramicrotome and thorough teaching of how to use the electron microscope. For all the great laughs and champagne parties with Ann Leich in the lab.

My co-author *Jian Kong* for holding the critical stabilizing sutures, caring for the cell cultures, the emergency sectioning and the all the good laughs.

My co-author Associate Professor *Peter Naeser* for his invaluable knowledge in histopathology.

Professor *Christine Curcio* for her advice on the difficult matter of BLIM's and BLAM's and her friendship.

Associate Professor *Giovanni Staurenghi* for setting my standards in ICG angiography and for all the nice stimulating discussions over feeder vessels.

Professor *Elke Lütjen-Drecoll* for her insight into the choroid and its nerve supply.

Professor *Gisele Soubrane* for considering "black holes" in ICG angiography.

Professor Rando Allikmets for interrupting my experiments with entertaining remarks when time was short…

Associate Professor *Takayuki Nagasaki* (Taka) always there with his computer genius and his ability to shrink the gigantic TIFF-illustrations just in time for publication.

Professor *Torvard* and *Ulla Laurent* for introducing me to the world of viscoelastics and the intriguing discussions about intraocular pressure…

Professor *Anders Bill* in his extraordinary knowledge involving intraocular circulation and the possibility of continuous IOP measurements, for allowing me to use the facilities in his laboratory during the night shift. I can never forget his valuable advice and Quiz ” Why beate a dead horse”.

Associate Professor *Per Törnquist* for just happen to come by, at the lab mentioned above, just in time before the rabbit was going to give in and helped me saving the whole experiment.

Professor *Endre Balazs* and *Janet Denlinger* for letting me work in their lab and introducing me to the owl monkey test and giving me the opportunity to perform intravitreal surgery in the cynomolgous monkeys essential for the outflow rate studies of exogenous hyaluronan. Introducing me to the extraordinary life in New York and generously entertaining in the city that “never sleeps”.

Michael Mercandetti and *Maurice Morose* who helped me feeding and caring for all these extra monkeys, the Nif –NaHa assays running until middle of the night and their disconcerting comments that “Lena means trouble; i.e. Lena means a lot of late work!”

In memory of Professor *Ernst Bárány* for his humor and the most important lessons on how to write an ethical application to get it approved.

In memory of Professor *David Maurice*, who worked on the same floor at Columbia, for his sly comments and his party spirit.

So now back to all the folks at S:t Eriks

Professor *Gunnar Lennerstrand* for his concern and interest in my research from a very early beginning at HS.

All the colleagues and staff in the department of anterior segment surgery with a lot of warm friendly feelings and support when time was difficult, always some nice supportive comments and *Örjan Wallin* offering a cup of cappuccino to cheer up the atmosphere. *Per* “*Monty*” *Montan* having his door open ready to give just the right remark and some refreshments. *Eva Lydahl* for her fantastic inspiration and memorable dinners in her kitchen. *Gisela Wejde* for the stimulating discussions late in the evening and all the good advice.

CG Laurell, “daddy” CG, former boss part time at the emergency ward, always calm with a lot of common sense and humor. Sharing the experience from KSK. Always ready to help out. Having an overall view from a high level.

Marie Ivarsson, the head nurse and the rest of the staff in the emergency ward including the night nurses, full of energy, knowing when and where, and soon to give support and positive remarks and a special thanks for the computer assistance by *Sanna Sundström & Sigrid Wikdahl. Lena Bååth* never missing a good sarcasm.

All the colleagues and staff in the department of medical and surgical retina including oncology. Professor *Stefan Seregard* always having a moment for a nice chat whenever the time is there and a lot of important advice. *Björn Steen* for lending me all the valuable intra-vitreal instruments that facilitated the critical surgical procedures. *Berit Carle-Petrelius* always quick in sending off a positive and caring e-mail. *Louise Bergman, Ann Odergren* and *Lotta All-Ericsson* for mental support. *Anders Kvanta* for being the positive external mentor, *Lennart Berglin* sharing the experience of the lab over there.

My “room mate” Eva Zand for putting up with all the papers and always there to give some positive and supportive remarks, “the little angel”.

To all the colleagues and staff in general ophthalmology and specially *Tomas Wijk, Eva Trané, Enping Chen, Bhupi Sawney* and *Frank Träisk* for all the supportive and friendly comments, as well as the folks in plastic surgery *Sven Sahlin* and *Eva Dafgård*.

Kristina Teär Fahnehjelm for all the encouraging help and advice over the years, late positive e-mail and sharing the experience of having a lot of children at home with all the knowledge of their demand.

And not to forget all the stimulating and inspiriting discussions with all our young residents.

Ulla Britt Schützler-Peterson for all her friendly help in practical matters and always making something extra just in case. Looking after so I get some more energy intake as a real “lab mother”. And not forget the friendly help from *Marianne Youssefi, Mimmi Wernman, Cecilia Danielson* and *Helene Asp* who helped to take me through the complicated bureaucracy of KI.

Fredrik Källmark for sharing the SLO world and lending me the important mouse.

Birgitta Groundstroem for always being ready to track down an immense number of references with great joy and *Ylva Lagerqvist* for her fantastic skill in making beautiful posters with short notification.

Associate professor *Bob Harris* who helped out, at late hours, to find the necessary important points for LADOK and all the practical matter of registration, despite everything he just made the impossible possible and in a times when his own little son had a severe cold. He encouraged me with This is NO PROBLEM; you will make it in time.

Professor *Martin Ingvar;* however pressed in time, spared a moment to fix the final signature and then had to make it all over again.

Associate Professor *Björn Ekesten* who had to really read this book and appreciate the work from the lab as we both share the same experience.

Agneta Rydberg, my dear old classmate, with all the enthusiasm and friendly remarks needed at the moments of doubts late at midnight with her usual cheerful comments; Of course I'm in the lab. It's just so fantastic that we all made it.

My dear friend Neurosurgeon *Kristina Cesarini* always being at the right spot, when need most, with all impossible and possible practical matter. Sharing the interest for research and stimulating discussions, at any time ready to cheer me up and vipe away any problems that had occured. Emergency babysitter and with an energy that is on a higher level than most.

Maria and Salomon Chelge who came "9/11" into the family for their fantastic friendship and caring for the kids when needed as mom always was continuously working late in trying to put this book into a reality.

And last but not least to my children; *Christopher, Patrik, Andreas, Svante, Anna* and *William*, who took the brunt of this adventure and made some of the original data, evaporate out into the cyber computer world - just reformatting the hard disc. Helping out in power point and paint. They always made me feel that I have to have the feet on the ground.

For financial support I would like to thank
The Crown Princess Margareta's Foundation for the Visually Handicapped
The Karolinska Institute (travel contribution)
Karin Sandqvist's Foundation
S:t Erik's foundation
Research to Prevent Blindness Inc.
NIH #EYO3854

13 REFERENCES

Acland GM, Aguirre GD, Bennett J, Aleman TS, Cideciyan AV, Bennicelli J, Dejneka NS, Pearce-Kelling SE, Magiure AM, Palczewski K, Hauswirth WW, Jacobson SG (2005) Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. Molecular Therapy 12:1072-1082

Algvere PV, Dafgard-Kopp EME, Gouras P (1999) Long-term outcome of RPE allografts in non-immunosuppressed patients. Eur J Ophthalmol 9:217-230

Alm A, Bill A (1972 a) The oxygen supply to the retina. I. Effects of changes in intraocular and arterial blood pressures, and in arterial P O2 and P CO2 on the oxygen tension in the vitreous body of the cat. Acta Physiol Scand. 84:261-274

Alm A , Bill A (1972 b) The oxygen supply to the retina II.Effects of high intraocular pressure and of increased arterial carbon dioxide tension on uveal and retinal blood flow in cats. Acta Physiol Scand 84:306-319

Alm A (2003) Ocular circulation. In: Kaufman P, Alm A (eds) Adler's Physiology of the Eye. Mosby, St Louis, section 13, chapter 6, pp 198-227

Archer D, Krill AE, Newell FW (1970) Fluorescein studies of normal choroidal circulation. Am J Ophthalmol 69:543-554

Baker KJ (1966) Binding if sulfobromophthalein (BSP) sodium and indocyanine green (ICG) by plasma α_1-lipoproteins. Proc Soc Exp Biol Med 122:957- 963

Ben Ezra D, Menigitsu F, Cividalli G, Weizman Z, Merin S, Auerbach E (1980) Chediak-Higashi syndrome: ocular findings. J Pediatr Ophthalmol Strabismus 17:68-74

Bennett J, Tanabe T, Sun D, Zeng Y, Kjeldbye H, Gouras P, Maguire AM (1996) Photoreceptor cell rescue in retinal degeneration (rd) mice by in vivo gene therapy. Nat med 2:649-654

Berglin L, Gouras P, Sheng Y, Lavid J, Lin PK, Cao H, Kjeldbye (1997) Tolerance of human fetal retinal pigment epithelium xenografts in monkey retina. Grafe's Arch Clin Exp Ophthalmol 235:103-110.

Berlin R (1873) Zur sogenannten commotio retinae. Klin Monatsbl Augenheilkd 1:42-78

Binder S, Stolba U, Krebs I, Kellner L, Jahn C, Feichtinger H, Povelka M, Frohner U, Kruger A, Hilgers RD, Krugluger W (2002) Transplantation of autologous retinal pigment epithelium in eyes with foveal neovascularization resulting from age-related macular degeneration: a pilot study. Am J Ophthalmol 133:215-225

Binder S. Krebs I, Hilgers RD, Abri A, Stolba U, Assadoulin A. Kellner I, Stanzel BV, Feichtinger H (2004) Outcome of transplantation of autologous retinal pigment epithelium in age-related macular degeneration: a prospective trial. Invest Ophthalmol Vis Sci 45:4151-4160

Bouchenaki N, Cimino L, Auer C, Tao Tran V, Herbort CP (2002) Assessment and classification in posterior uveitis using indocyanine green angiography. Klin Monatsbl Augenheilkd 219:243-249

Burns RP, Feeney-Burns L (1980) Clinico-morphologic correlations of drusen of Bruch's membrane. Trans Am Ophthalmol Soc 78:206-215

Cioffi GA, Alm A (2001) Measurement of ocular blood flow J Glaucoma 10(Suppl 1):S62-S64

Collier LL, Prieur DJ, King EJ (1984) Ocular melanin pigmentation anomalies in cats, cattle, mink and mice with Chediak-Higashi syndrome: histologic observations. Curr Eye Res 3:1241-1251

Collier LL, King EJ, Prieur DJ (1986) Age-related changes of the retinal pigment epithelium of cats with Chediak-Higashi syndrome. Invest Ophthalmol Vis Sci 27:702-707

Crafoord S, Algvere PV, Seregard S, Kopp ED, (1999) Long-term outcome of RPE allografts to the subretinal space of rabbits. Acta Ophthalmol Scand 77:247-254

Crafoord S, Algvere PV, Kopp ED, Seregard S (2000 a) Cyclosporine treatment of RPE allografts in the rabbit subretinal space of rabbits. Acta Ophthalmol Scand 78:122-129

Crafoord S, Kopp ED, Seregard S, Algvere PV (2000 b) Cellular migration into neural retina following implantation of melanin granules to the subretinal space. Graefes Arch Clin Exp Ophthalmol 238:682-689

Crafoord S, Geng L, Seregard S, Algvere PV (2001) Experimental transplantation of autologous iris pigment epithelial cells to the subretinal space. Acta Ophthalmol Scan 79:509-514

Cuthbertson S, Jackson B, Toledo C, Fitzgerald MEC, Shih VF, Sagvazdin YS, Reiner A (1997) Innervation of orbital and choroidal blood vessels by the pterygopalatine ganglion in pigeons. J Comp Neurol 386:422-442

Del Priore LV, Hornbeck R, Kaplan HJ, Jones Z, Valentino TL (1995) Debridement of the pig retinal pigment epithelium in vivo. Arch Ophthalmol 113:939-944

Del Priore LV, Kaplan HJ, Hornbeck R, Jones Z, Swinn M (1996) Retinal pigment epithelial debridement as a model for the pathogenesis and treatment of macular degeneration. Am J Ophthalmol 122:629-643

Del Priore LV, Tezel TH (1998) Reattachment rate of human retinal pigment epithelium to layers of Human Bruch's membrane. Arch Ophthalmol 116:335-341

Erickson AC, Couchman JR (2000) Still more complexity in mammalian basement membranes. J Histochem Cytochem 48:1291-1306

Feng W, Yasumura D, Matthes MT, La Vail MM, Vollrath D (2002) Mertk triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells. J Biol Chem 277:17016-17022

Fisher SK, Anderson DH (1998) Cellular responses of the retinal pigment epithelium to retinal attachment and reattachment. Chapter 20 pp.406-419 in The Retinal Pigment Epithelium: Function and Disease (Marmor MF, Wolfensberger TJ, eds) Oxford University Press.

Fischmeister R, Hartzell HC (2005) Volume sensitivity of the bestrophin family of chloride channels. J Physiol 15:477-491

Flower RW, Hochheimer BF (1973) A clinical technique and apparatus for simultaneous angiography of the separate retinal and choroidal circulation. Invest Ophthalmol 12:248-261

Flugel C, Tamm ER, Mayer B, Lutjen-Drecoll E (1994) Species differences in choroidal vasodilative innervation: Evidence for specific intrinsic nitrergic and VIP-positive neurons in the human eye. Invest Ophthalmol Vis Sci 35:592-599

Flugel-Koch C, May CA, Lutjen-Drecoll E (1996) Presence of a contractile cell network in the human choroid. Ophthalmologica 210:296-302

Foulds WS, Lee WR, Taylor WO (1971) Clinical and pathological aspects of choroidal ischaemia Trans Ophthalmol Soc UK 91:323-341

Gallemore RP, Hughes BA, Miller BS (1997) Retinal pigment epithelial transport mechanisms and their contributions to the electroretinogram. Prog Retin Eye Res 16: 509-599

Ghosh F, Johansson K, Ehinger B (1999) Long-term full-thickness embryonic rabbit retinal transplants. Invest Ophthalmol Vis Sci 40:133-142

Gjörloff K, Bruun A, Ghosh F (2001) Cone photoreceptors in laminated retinal transplants. Acta ophthalmol Scand 79:366-369

Gouras P, Du J, Kjeldbye H, Yamamoto S, Zack DJ (1994) Long-term photoreceptor transplants in dystrophic and normal mouse retina. Invest Ophthalmol Vis Sci 35:3135-3145

Gouras P, Kong J, Tsang SH (2002) Retinal degeneration and RPE transplantation in Rpe65 -/- mice. Invest Ophthalmol Vis Sci 43:3307-3311

Gouras P. (2004) What are the problems with retinal cell transplants? In The Macula (S. Binder, ed.) Springer, Vienna.

Gouras P, Ivert L, Mattison J, Neuringer M (2006) Drusenoid retinopathy in rhesus monkeys (Macaca Mulatta). Invest Ophthalmol Vis Sci # 856

Guyer DR, Yannuzzi LA, Slakter JS, Sorenson JA, Hanutsaha P, Spaide RF, Schwartz SG, Hirschfeld SG, Orlock DA (1996) Classification of choroidal neovascularization by digital indocyanine green videoangiography. Ophthalmology 103:2054-2060

Haefeli WE, Bargetzi MJ, Starnes HF, Blashke TR, Hoffman BB (1993) Evidence for activation of the sympathetic nervous system by recombinant human interleukin-1 beta in humans. J Immunother 13:136-140.

Hageman GS, Luthert PJ, Chong NHV, Johnson LV, Anderson,DH, Millins RF (2001) An intergrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog Ret Eye Res 20:705-732

Hayreh SS (1975) Segmental nature of the choroidal vasculature. Br J Ophthalmol 59:631-648

Hayreh SS (2004) Posterior ciliary artery circulation in health and disease. The Weisenfeld Lecture. Invest Ophthalmol Vis Sci 45:749-757

Herriot WJ, Machemer R (1992) Pigment epithelial repair. Graefes Arch Clin Exp Ophthalmol 230:230-291

Hirata Y, Nishiwaki H, Miura S, Ieki Y, Kiryu J, Honda Y (2003) In vivo analysis of choroidal circulation by continuous laser-targeted angiography in the rat. Invest Ophthalmol Vis Sci 44:3103-3109

Hogan MJ, Alvarado JA, Weddell JE (1971) Histology of the human eye. Saunders Co, Philadelphia.

Hollenberg MJ, Burt WL (1969) The fine structure of Bruch's membrane in the human eye. Canad J Ophthal 4:296-306

Hughes BA, Gallemore RP, Miller SS (1998) Transport mechanisms in the retinal pigment epithelium. Chapter 6 pp.103-134 in The Retinal Pigment Epithelium: Function and Disease (Marmor MF, Wolfensberger TJ, eds) Oxford University Press.

Iijima H, Ida T, Murayama K, Imai M, Gohdo T (1999) Plasminogen activator inhibitor 1 in central serous chorioretinopathy. Am J Ophthalmol 127:477-488

Immel J, Negi A, Marmor MF (1986) Acute changes in RPE apical morphology after retinal detachment in rabbit. A SEM study. Invest Ophthalmol Vis Sci 27:1770-1776

Ishibashi T, Sorgente N, Patterson R, Ryan SJ (1986) Pathogenesis of drusen in primate. Invest Ophthalmol Vis Sci 27:184-193

Ivert L, Gouras P, Naeser P, Narfstrom K. (1998) Photoreceptor allografts in a feline model of retinal degeneration. Graefes's Archive Clin Exp Ophthalmol 236:844-852

Ivert L, Kjeldbye H, Gouras P (2002) Long term effects of short term retinal bleb detachments in rabbits.Graefe's Arch Clin Exp Ophthalmol 240:232-237

Ivert L, Kong J, Gouras P (2003) Changes in the choroidal circulation of rabbit following RPE removal. Graefe's Arch Clin Exp Ophthalmol 241:656-666

Ivert L, Kjeldbye H, Gouras P (2005) Age related changes in the basement membrane of the retinal pigment epithelium of RPE65 -/- and wild type mice. Graefe´s Archive Clin Exp Ophthalmol 243:250-256

Kaplan HJ, Tezel TH, Berger AS, Wolf ML, Del Priore LV (1997) Human photoreceptor transplantation in retinitis pigmentosa: A safety study. Arch Ophthalmol 115:1168-1172

Kaplan HJ, Tezel TH, Del Priore LV (1998) Retinal pigment epithelial transplantation in age-related macular degeneration (editorial). Retina 18:99-102

Kitaya N, Nagaoka T, Hikichi T, Sugawara R, Fukui K, Ishiko S, Yoshida A (2003) Features of abnormal choroidal circulation in central serous chorioretinopathy. Br J Ophthalmol 87:709-712

Kong J, Pata I, Kim SR, Binley K, Naylor S, Leboulch P, Sparrow JR, Gouras P, Allikmets R (2005) Correction of the disease phenotype in the mouse model of Stargardt disease by Lentiviral gene therapy. Invest Ophthalm Vis Sci # 5210

Korte GE, Reppucci V, Henkind P (1984) RPE destruction causes choriocapillary atrophy. Invest Ophthalmol Vis Sci 25:1135-1145

Krey HF (1975) Segmental vascular patterns of the choriocapillaris. Am J Ophthalmol 80:198-202

Kroll AJ, Machemer R (1969) Experimental retinal detachment in the owl monkey V. Electron microscopy of the reattached retina. Am J Ophthalmol 67:117-130

Lai C, Gouras P, Doi K, Tsang SH, Goff SP, Ashton P (2000) Local immunosuppression prolongs survival of RPE xenografts labeled by retroviral gene transfer. Invest Ophthalmol Vis Sci 41:3134-3141

Leonard DS, Zhang X, Panozzo G, Sugino IK, Zarbin MA (1997) Clinicopathological correlation of localized retinal pigment epithelium debridement. Invest Ophthalmol Vis Sci 38:1094-1109

Li L, Turner JE (1988) Inherited retinal dystrophy in the RCS rat: Prevention of photoreceptor degeneration by pigment epithelial cell transplantation. Exp Eye Res 47:911-917

Lopez R, Gouras P, Kjeldbye H, Sullivan B, Reppucci V, Brittis M, Wapner F, Goluboff E (1989) Transplanted retinal epithelium modifies the retinal degeneration in the RCS rat. Invest Ophthalmol Visc Sci 30:586-588

Lopez PF, Yan Q, Kohen L, Rao NA, Spec C, Black J, Oganesian A (1995) Retinal pigment wound healing in vivo. Arch Ophthalmol 113:1437-1446.

Luscher TF, Yang Z, Tschudi M, von Segesser L, Stulz P, Boulanger C, Siebenmann R, Turina M, Buhler FR (1990) Interaction between endothelin-1 and endothelium-dervied relaxing factor in human arteries and veins. Circ Res 66:1088-1094.

Machemer R (1968) Experimental retinal detachment in the owl monkey. II. Histology of retina and pigment epithelium. Am J Ophthalmol 66:396-410

Machemer R, Norton EWD (1968) Experimental retinal detachment in the owl monkey: I. Methods of production and clinical picture. Am J Ophthalmol 66:388-396

Machemer R, Laqua H (1975) Pigment epithelial proliferation in retinal detachment (massive periretinal proliferation). Am J Ophthalmol 80:1-23

Machemer R, van Horn D, Aaberg TM (1978) Pigment epithelial proliferation in human retinal detachment with massive periretinal proliferation. Am J Ophthalmol. 85:181-191

Marmor MF, Wolfensberger TJ, eds. (1998) The retinal pigment epithelium. Function and disease. New York: Oxford 103-134

May CA, Horneber M, Lutjen-Drecoll E (1996) Quantitative and morphological changes of the choroid vasculature in RCS rats and their congenic controls. Exp Eye Res 63:75-84

Nakaizumi Y (1964) The Ultrastructure of Bruch's membrane I-III. Arch Ophthalmol 72:380-400

Nilsson SFE, Linders J, Bill A (1985) Characteristics of uveal vasodilation produced by facial nerve stimulation in monkeys, cats, and rabbits. Exp Eye Res 40:841-852

Olver JM (1990) Functional anatomy of the choroidal circulation; methyl methacrylate casting of human choroid. Eye 4:262-272

Ozaki S, Yamana T, Kita M, Negi A, Honda Y (1995) The reorganization process of the retinal pigment epithelium-photoreceptor interface for 6 months observation after RPE removal. Invest Ophthalmol Vis Sci 36 (4):S252, 1143

Parolini B, Sugino IK, Gordon E, Zarbin MA (1995) A new method to debride RPE cells from Bruch's membrane in vitro. Invest Ophthalmol Vis Sci 36 (4):S252, 1141

Paumgartner G (1975) The handling of indocyanine green by the liver. Schweiz Med Wochenschr 105 (suppl) 17:1

Petrukhin K, Koisti MJ, Bakall B, Li W, Xie G, Marknell T, Sandgren O, Forsman K,Holmgren G, Andreasson S, Vujic M, Bergen AA, McGarty-Dugan V, Figueroa D, Austin CP, Metzker ML, Caskey CT, Wadelius C (1998) Identification of the gene responsible for Best macular dystrophy. Nat Genet 19:241-247

Piccolino FC, Borgia L, Zinicola E, Zingirian M (1995) Indocyanine green angiographic findings in central serous chorioretinopathy.Eye 9:324-332

Prunte C, Flammer J (1996) Choroidal capillary and venous congestion in central serous chorioretinopathy. Am J Ophthalmol 121:26-34

Ramirez JM, Trivino A, De Hoz R, Ramirez AI, Salazar JJ, Garcia-Sanchez J (1999) Immunohistochemical study of rabbit choroidal innervation. Vision Research 39: 1249-1262

Robison WG, Kuwabara T, Cogan DG (1975) Lysosomes and melanin granules of the retinal pigment epithelium in a mouse model of the Chediak-Higashi syndrome. Invest Ophthalmol Vis Sci 14:311-317

Robison WG, Kuwabara T (1978) A new albino-beige mouse; giant granules in retinal pigment epithelium. Invest Ophthalmol Vis Sci 17:365-370

Salchow DJ, Trokel SL, Kjeldbye H, Dudley T, Gouras P (2001) Isolation of human fetal cones. Curr Eye Res 22:85-89

Sayangi K, Fujikado T, Onodera T, Tano Y (2003) Chediak-Higashi syndrome with progressive visual loss. Jap J Ophthalmol 47:304-306.

Schneider U, Inhoffen W, Gelisken F (2003) Indocyanine green angiography in a case of recurrent posterior acute multifocal placoid pigment epitheliopathy. Acta Ophthalmol Scand 81:72-75

Seiler MJ, Aramant RB, Ball SL (1999) Photoreceptor function of retinal transplants implicated by light-dark shift of S-antigen and rod transducin. Vision Research 39:2589-2596

Shiflett SL, Kaplan J, Ward DM (2002) Chediak-Higashi Syndrome: a rare disorder of lysosomal and lysosome related organelles. Pigment Cell Res 15:251-257

Shimizu K, Ujiie K (1978) Structure of ocular vessels. Igaku-Shoin Ltd, Tokyo

Shimizu K, Ujiie K (1981) Morphology of the submacular choroids: Vascular structure. Ophthalmol 183:5-10

Tezel TH, Kaplan HJ, Del Priore LV (1999) Fate of human retinal pigment epithelial cells seeded onto layers of human Bruch's membrane. Invest Ophthalmol Vis Sci 40:467-476

Torczynski E, Tso MO (1976) The architecture of the choriocapillaris at the posterior pole. Am J Ophthalmol 81:428-440

Trivino A, de Hoz B, Rojas JJ, Salazar JJ, Ramirez AI, Ramirez JM (2005) NPY and TH innervation in human choroidal whole-mounts. Histol Histopathol 20:393-302

Valentino TL, Kaplan HK, Del Priore LV, Fang S, Berger A, Silverman MS (1995) Retinal pigment epithelial repopulation in monkeys after submacular surgery. Arch Ophthalmol 113:932-938

Valenzuela R, Morningstar WA (1981) The ocular pigmentary disturbance of human Chediak-Higashi syndrome. Am J Clin Pathol 75:591-596

Van Meurs JC, Van Den Biesen PR (2003) Autologous retinal pigment epithelium and choroid translocation in patients with exudative age-related macular degeneration: short-term follow-up. Am J Ophthalmol 136:688-69

Wang H, Leonhard DS, Castellarin AA, Tsukahara I, Ninomiya Y, Yagi F, Cheewatrakoolpong I, Sugino IK, Zarbin MA (2001) Short-term study of allogeneic retinal pigment epithelium transplants onto debrided Bruch's membrane. Invest Ophthalmol Vis Sci 42:2990-2999

Ward DM, Griffiths GM, Stinchcombe JC, Kaplan J (2000) Analysis of the Lysosomal Storage Disease Chediak-Higashi Syndrome. Traffic 1:816-822.

Weiter JJ, Ernst JT (1974) Anatomy of the choroidal vasculature. Am J Ophthalmol 78:583-590

Wilson CA, Choromokos EA, Sheppard R (1988) Acute posterior multifocal placoid pigment epitheliopathy. Brit J Ophthalmol 73:985-990

Yamamoto S, Du J, Gouras P, Kjeldbye H (1993) Retinal pigment epitheilial transplants and retinal function in RCS rats. Invest Ophthalmol Vis Sci 34:3068-3075)

Yoneya S, Tso MO (1987) Angioarchitecture of the human choroid. Arch Ophthalmol 105:681-687

Zhang XY, Bok D (1998) Transplantation of retinal epithelial cells and immune response in the subretinal space. Invest Ophthalmol Vis Sci 30: 1021-1027

Zrenner C (1984) The discovery of the lamina basalis choroideae by Carl Wilhelm Ludvig Bruch (1819-1884). Hist Ophthal Intern 3:213-260